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Neuroprotective effects of Complement C3 convertase inhibition after Traumatic brain injury a randomized placebo-controlled study in mice

INAUGURAL-DISSERTATION

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1. Summary

Despite the fact that neurological diseases of other etiologies receive more attention by the media, biomedical research and supporting foundations in industrialized countries, TBI still remains the leading cause of death in young individuals up to the age of 40 years. Research efforts in the past decade have focused on elucidating the role of the massive intracranial inflammatory response in the injured brain with regard to the neuropathological sequelae which are, in large part, responsible for the adverse outcome in neurotrauma patients. In the present study, we have investigated the effect of pharmacological complement inhibition at the C3 convertase (by Crry-Ig) on apoptosis, neuronal cell death and complement activation after experimental head injury, with a special focus on the anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein.

After trauma, the genes for C1-Inh and CD55 were significantly upregulated within 4h in the *Crry-Ig*-injected group, as compared to vehicle-injected controls. The genes' expression was assessed using a semi quantitative two-step real-time RT-PCR. The CD59 gene showed a slightly differing kinetic with a peak at 24 h, whereas the CD55 gene remained significantly elevated above the vehicle controls for up to 7 days after TBI. In contrast, the C1-Inh gene was significantly suppressed at the later time-points (24h and 7 days) compared to vehicle-injected mice. These data suggest that *Crry-Ig* modulates its anti-complement effects not only by direct inhibition of C3 convertases.

We identified a posttraumatic upregulation of Bcl-2 mRNA within 7 days, which was further induced in the *Crry-Ig*-treated group as early as 4h after trauma associated by an increase in intracerebral Bcl-2 expression, which was confirmed at the protein level by Western blot analysis. Interestingly, Bax mRNA was also provoked after introduction of Crry-Ig and Bax expression was increased but the Bax/Bcl-2 ratio was suggestible in favour of neuroprotection. This was confirmed by anti NeuN antibody stain which revealed neuroprotective signs in hippocampus and TUNEL histochemistry showing lower number of TUNEL positive cells in Crry-Ig treated mice vs. vehicle-treated mice.

In conclusion, an effective pharmacological inhibition of the intracerebral complement activation, should provide a future avenue for successful attenuation of secondary brain injury, and may hopefully help reduce the high rate of neurological morbidity and delayed mortality in neurotrauma patients.

2. Introduction

2.1 Traumatic Brain Injury

Since several death and disability cases are reported after traumatic brain injury (TBI), it is regarded as a major public problem and the number of brain injuries sustained each year has been subjected to intense study with an overall incidence of 235 per 100 000 persons per year[2].

Kraus and co-workers, depending on national and international studies calculated the average brain injury death rate at about 22 per 100,000 per year [3]. In industrialised nations, TBI represent the leading cause of death and residual logical impairment in young patients under the age of 40 years[4-9]. In the states of the Union approximately 500,000 patients with TBI require hospitalization every year and mostly young people with a median age of 25 years are affected [4-6, 10, 11]. It's also to be considered that about half of all trauma deaths in the United States involve significant injury to the brain[12]. The direct and indirect costs in the United States are estimated to be about 60 billion dollars [13-15]. Despite advances in research and improved neurointensive care in the last decade, the clinical outcome of several head-injured patients is still poor and the mortality rate remains as high as 35-40% [4, 16, 17].

The follow-up study from Koponen and co-workers revealed that the up to 50-60% of the TBI patients are susceptible to psychiatric disorders, consequently underlying the high socio-economic impact of head injury [17].

The extent of residual brain damage is determined by the primary and secondary injuries. The primary injury to the central nervous system (CNS) is due to the mechanical forces applied to the skull and brain at the time of impact, leading to focal or diffuse injury patterns, whereby the secondary injuries are a result of complicated processes initiated by the primary insult and lead to blood brain barrier (BBB) dysfunction, induction of cerebral edema and intracranial hypertension [7, 16, 18-20].

Evidence of secondary brain injury has been found at autopsy in 70%-90% percent of all fatally head-injured patients[5]. These Secondary insults induce neuroinflammation by activation of the innate immunity, e.g. through complement activation, therefore triggering a profound host-mediated inflammatory response within the intracranial compartment[21-26]. Among the crucial endogenous mediators of neuroinflammation are pro-inflammatory cytokines [27-31], chemokine [28] and complement anaphylatoxins [32-34] which induce chemotaxis of blood-derived leukocytes across the BBB into the subarachnoid space (SAS) [21, 35-37]. On one hand, these recruited inflammatory cells exacerbate and

perpetuate the inflammatory response in the CNS which further contribute to the development of secondary brain damage, e.g. through the oxidative burst of neutrophils associated with the release of proteolytic and neurotoxic enzymes[38-42] or hypoxia and hypotension which cause cerebral ischemia and reperfusion injuries[43]. On the other hand, the inflammatory response of the injured brain seems to induce beneficial effects as well, in terms of post-traumatic local induction of neurotrophic factors[44, 45]. Clinical and experimental studies have revealed that pro-inflammatory cytokines, such as interleukin (IL)- 1β [44, 46-48], IL-6[49, 50] and IL-8[51] are able to induce the production of the neurotrophin nerve growth factor (NGF) within the injured CNS. The net balance between detrimental and beneficial aspects of cerebral inflammation after TBI remains to be elucidated[27, 30, 47]. The expanding knowledge of the basic cellular and molecular mechanisms responsible for the inflammatory response of the injured brain has evoked new therapeutic approaches for head-injured patients[16, 52-56]. However, no efficacious pharmacotherapy has been developed for patients with severe TBI to date[5, 45, 57, 58]. As long as we can not influence the primary injury, the main target in the treatment of patients is the optimization of therapy to reduce the secondary injury. The aim of the experimental traumatologie is to develop, try and prove the effect of the potentially neuroprotective substances which would influence this process.

2.2 Inflammatory brain response after trauma

A traumatic impact to the brain stimulates metabolic and inflammatory processes which exacerbate the primary traumatic injury to neurons, leading to secondary brain damage [7, 24, 26, 54, 59]. The primary injury is the mechanical force applied to the skull causing local or diffuse brain injury , such as diffuse axonal injury (DAI), cerebral contusion, laceration of the neurons and intracranial bleeding [10, 16, 60-63]. The metabolic and immunological reaction leads to an extended intracranial inflammatory process and secondary injury which is believed to be responsible for the poor outcome after TBI [64]. This secondary brain injury occurs as a consequence of complicating processes initiated by the primary injury and is characterized by neuroinflammation, ischemia/reperfusion injuries, cerebral edema, intracranial hemorrhage, and intracranial hypertension (**Fig. 1**) [65, 66]. The development of brain edema as a result of the secondary injury plays a big role in the poor prognosis after sever TBI [18, 67]. The neuroinflammatory reaction leads to a secondary cerebral ischemia which increases the

inflammatory reaction and cell death [68]. The early hypoxia and hypotension are as well important risk factors “lethal duo” for the exaggeration of the immune reaction by significantly decreasing the extent of brain oxygenation and cerebral perfusion due to shock and pulmonary injuries[43, 62, 69]. Additionally the peripheral injuries like open fractures could activate the immune defense and boost the inflammatory reaction.

Cerebral endothelial injury is a concomitant of head injury[70]. The mechanism is not yet clear but its thought to be a result of direct or indirect injury, such as impact on cerebral vessels, hemodynamic stress, hypoxia, cerebral ischemia, or brain edema [71].

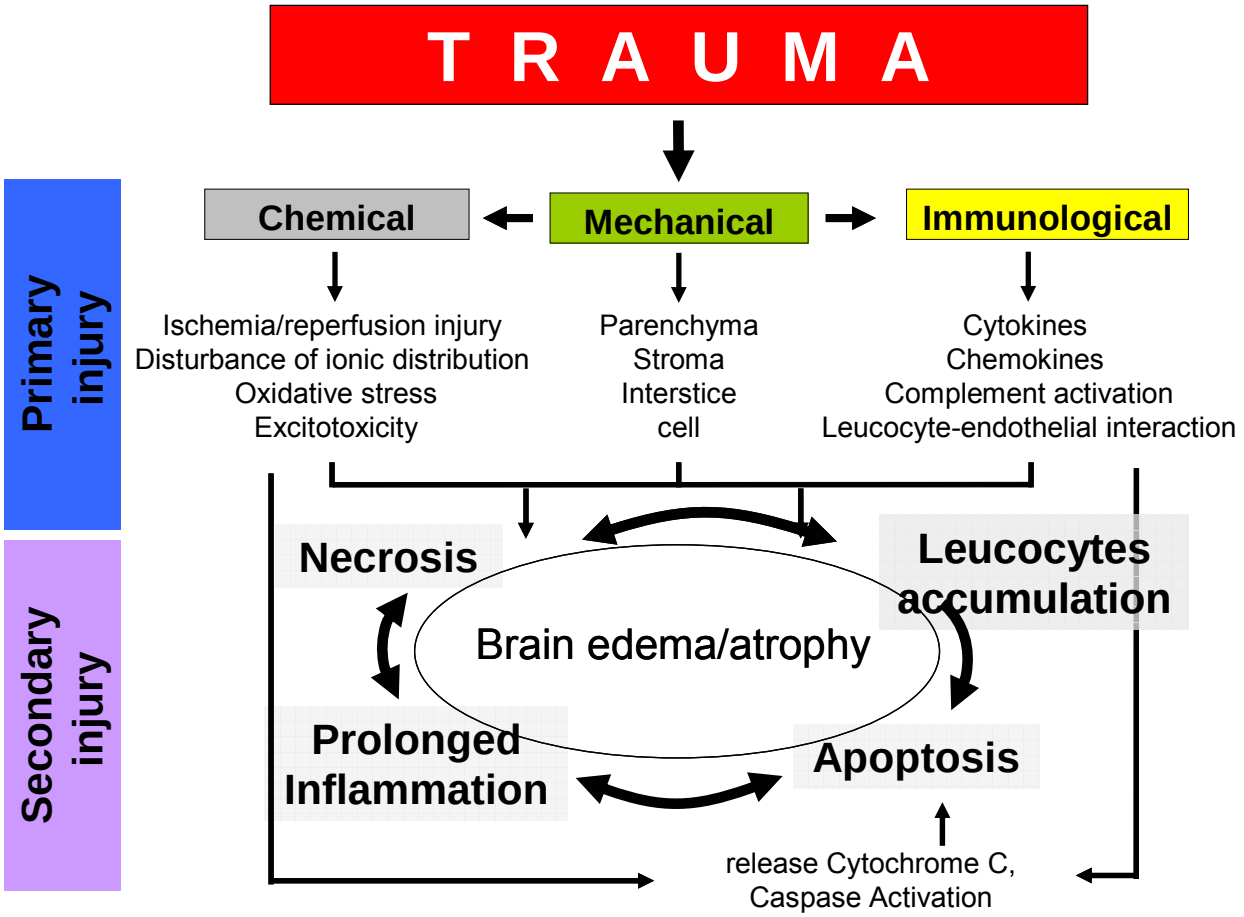

Figure 1. Primary and secondary injuries after TBI. The primary injury is the mechanical force applied to the skull causing local or diffuse brain injury, the secondary brain injury occurs as a consequence and has a chemical and neurological components. It is characterized by neuroinflammation, ischemia/reperfusion injuries, apoptosis, necrosis, brain edema and atrophy.

Cerebral endothelial injury or activation promotes the release of inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β [22, 25, 26, 30, 72, 73], and the promotion of an inflammatory process in the cerebral endothelium may amplify the endothelial injury or activation[74]. This contributes to the BBB damage, since these cytokines have been shown to induce BBB dysfunction and intracranial inflammation in

a variety of experimental models [75-77]. This BBB disturbance lead to passive leakage of serum proteins into the intrathecal compartment [78-82]. Injured endothelial cells lose their ability to maintain a balance between coagulation and anticoagulation factors, to regulate the tone of smooth muscle, and to control vascular permeability. Increased vascular permeability in the cerebral parenchyma may result in delayed traumatic intracerebral hematoma[71], induction of vasogenic brain edema, leading to increased intracranial pressure (ICP) and decreased cerebral perfusion pressure (CPP), thus aggravating cerebral ischemia and the concomitant pathophysiological events [80].

The CNS has often been considered an “immunologically privileged site”, this assumption being based on the absence of lymphatic drainage and the unique type of endothelium forming the BBB. There is no doubt that the brain differs significantly from other tissues in its responses to pathogenic challenges. Infection or inflammation elicits rather different responses in the brain to those in other tissues. This is most evident in leukocyte recruitment, which is rapid in many systemic organs, but modest and delayed in the brain [83]. Post-traumatic, blood-derived leukocytes are attracted across the BBB into the Subarachnoid space (SAS) [21, 26], microglia and blood-derived macrophages are activated by CNS damage or infection, and are recruited rapidly to the site of insult [83]. The presence of damaged cells and debris causes ramified resting microglia to transform into rounded migratory macrophages and produce cytokines and trophic factors that can exert damaging or protective effects on neighbouring cells [83]. The recruitment of neutrophils into the CNS is particularly important for the inflammatory response of the injured brain, since neutrophils have been shown to significantly contribute to host tissue damage by release of proteolytic enzymes and reactive oxygen intermediates [42, 84].

The data suggest that, even under normal conditions, there is a moderate traffic of hematogenous cells through the BBB [85, 86]. The presence of astrocytes and microglia that embody the immune function, and the restrictive BBB, which is also known to possess immune functions, argue strongly for the unique immune status of the CNS [87]. Neurones, astrocytes, microglia and oligodendrocytes can produce inflammatory mediators, and cytokine receptors are expressed constitutively throughout the CNS, albeit at low levels. The constitutive expression of genes encoding cytokines and their receptors in the brain suggests that cytokines may contribute to normal physiological functions of the CNS [83]. It is now clear that the CNS is characterized by only a partial

immune privilege, in terms of downregulation and suppression of many aspects of the immune function in comparison with other organs [88].

Experiments with T-cell lines specific for CNS antigens have led to the surprising conclusions that the CNS is routinely surveyed by activated T-lymphocytes that can cross the BBB, and that astrocytes play a major part in the initiation and subsequent regulation of the intracerebral immune response [89, 90]. Since microglia cells have been shown to produce inflammatory mediators (cytokines), acting as antigen-presenting cells for T-lymphocytes, these cells may play a fundamental role in the induction and maintenance of the inflammatory process in the CNS following a closed head injury [91, 92].

Induction of programmed cell death (PCD; apoptosis) in neurons has been demonstrated in experimental models of TBI[93-96] . In addition, mediators of PCD, such as soluble Fas, soluble Fas ligand (FasL), and caspase-3, have been detected in elevated amounts in the CSF of TBI patients[97-99]. Fas is of particular interest because it belongs to the TNF receptor superfamily; both share common steps of the downstream pathway, including the activation of caspases, pivotal factors in programmed cell death. Fas and FasL have been shown to be expressed on mouse neurons after experimental TBI[100] and their expression overlaps with neurons undergoing posttraumatic cell death [101]

2.3 Cytokines and brain injury

Cytokines are low molecular weight proteins which act as mediators of ‘‘communication’’ between leukocytes, bridging innate and adaptive immune responses. Pro-inflammatory cytokines, such as TNF and the IL-1b, -6, -8, -12, -15, and -18 have been described as potent inflammatory mediators induced by traumatic tissue injuries [102-104].

The TBI is believed to induce the local synthesis of cytokines by resident cells in the CNS [28, 30, 105-107] and to activate the innate immunity through the activation of the complement system [108] within seconds to minutes. The complement anaphylatoxins C3a and C5a triggers the induction of high potent pro-inflammatory cytokines, e.g. IL-1β or TNF. A delayed activation of other pro-inflammatory cytokines e.g. IL-6, IL-18 and chemokines occurs within hours to days intracranially [48,109]. Cytokines can also cross the BBB, probably either by active transport or through leaky regions of endothelia when the BBB is compromised by a pathological condition [83]. Therefore the CNS is liable for affection by central as well as peripherally produced inflammatory mediators.

These mediators lead to the inflammatory reaction within the injured brain known as neuroinflammation [63]. Clinical studies have demonstrated elevated levels of TNF, IL-6, and soluble IL-6 receptor in CSF and serum from head trauma patients [110-115]. Elevated levels of IL-12 and IL-18, pro-inflammatory cytokines with IFN (interferon) -γ modulating properties were also demonstrated in human CSF of patients with severe TBI[116, 117]

The proinflammatory cytokines IL-1, IL-1α und IL-1β are significant mediators for the intracranial inflammatory response [48, 83, 107, 118, 119]. The signal transduction takes place through the 80 kDa- IL1 membrane Receptor (IL-1R) in which the competitive and high selective antagonist IL-1 Ra binds [120]. Normally IL-1α and IL-1β are hardly detected in brain[119]. Following trauma there is a fast induction of IL-1α and IL-1β gene expression in the glia cells as well as neurons and the cerebral vascular system's endothelial cells [48, 119, 121-124]. IL-1 has a wide pro inflammatory effect which includes the induction of the central complement factor C3, nitrogen oxide synthase (NOS), the cyclooxygenase-2(COX-2) and other pro-inflammatory cytokines (e.g. IL-6 and IL-8) through multiple immune competent central and peripheral cells (**Fig. 2**) [48, 119, 125, 126]. An experimental study using trasgenetic mice with IL1Ra over expression showed that the inhibition of IL-1 lead to significant improvement of the neurological outcome following TBI [120]. The determining action of IL-1 is the induction of apoptosis in the CNS [118, 127]. Another indirect function of IL-1α is the induction of vascular endothelial growth factor (VEGR) [128], which is an important mediator in the post traumatic brain edema [129] (**Fig. 2**). In this way, IL-1 contributes through different molecular events to the cell death in the affected brain.

TNF can be considered a major pro-inflammatory cytokine with an optional capacity to induce apoptosis [130]. TNF can be active both as a membrane integrated [130] and a soluble molecule after its processing by the metalloprotease TACE (TNFα-converting enzyme) [131]. This cytokine is reported to have both deleterious and protective actions in neurons, and these opposing effects may be explained by the existence of two distinct TNF-signaling pathways mediated by two receptors, p55 and p75 (**Fig. 2**) [73]. The p75 in the brain contains TRAF (TNF receptor-associated Factors)-interacting motifs that recruit TRAFs and thus directly activate the same intracellular pathways as IL-1 [132]. Activation of p55 initiates signals leading to neuronal apoptosis [133]. In the CNS, resident macrophages, astrocytes and microglia are able to synthesize TNFα,

which seems to be proinflammatory during the acute phase of CNS inflammatory responses, but immunosuppressive during the chronic phase [83]. The duality of the role of TNFα has been elegantly demonstrated in mice lacking this cytokine: acutely after cortical impact injury, the TNFα knockout mice show less behavioural impairment than the wild-type controls, but while the wild-type mice recovered in the weeks following injury, the knockout mice did not, remaining significantly impaired both in terms of histological damage and behaviour [134]. These divergent activities may arise from the existence of two TNFα receptors.

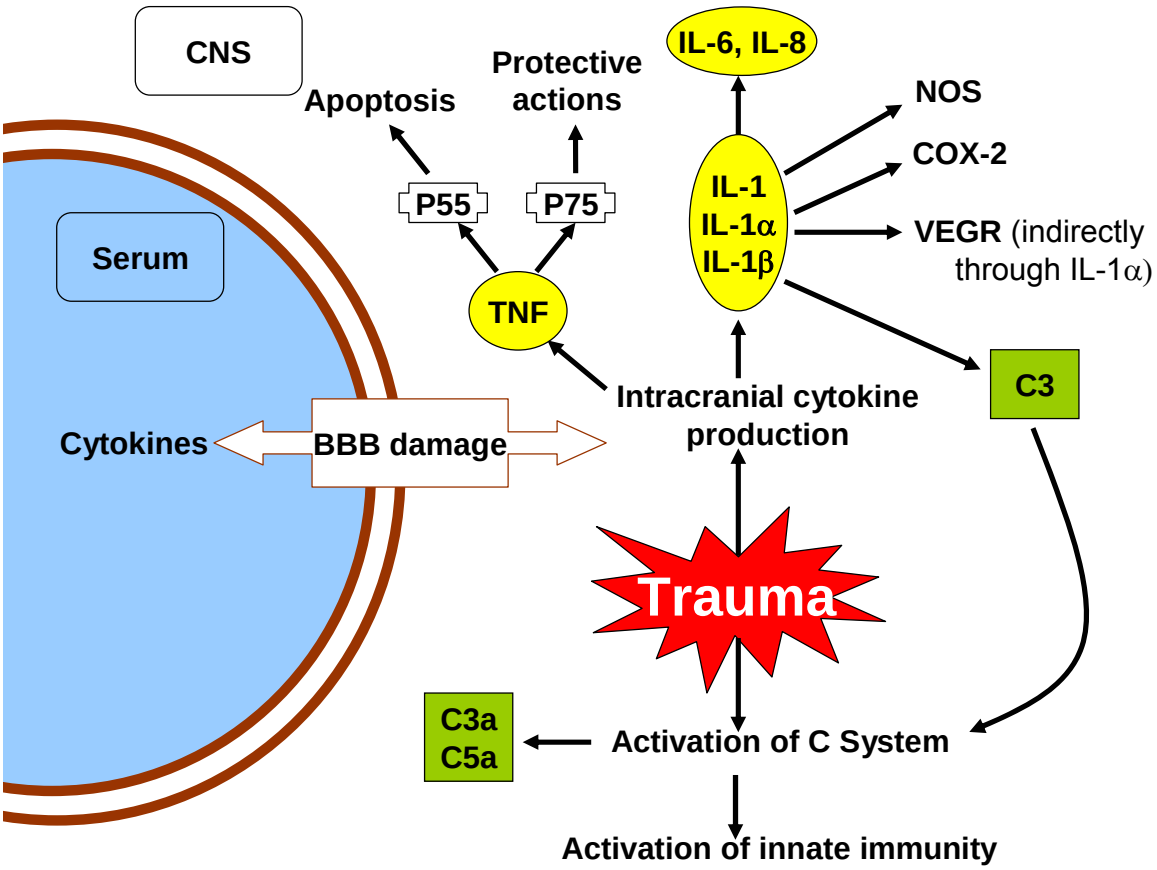

Figure 2. The release of cytokines after TBI. TBI induces the local synthesis of cytokines in CNS. The proinflammatory cytokines IL-1, IL-1α und IL-1β have a wide pro inflammatory effect which includes the induction of C3, NOS, COX-2, IL-6, IL-8 and VEGR indirectly. See text for abbreviations

The simultaneous pro- and anti-inflammatory effect mediated by many of the ‘‘key’’ inflammatory agents, such as TNF, implies that the irreversible pharmacological blocking may also lead to unexpected adverse events [30]. Due to central role of proinflammatory cytokines in the pathophysiology of severe injuries, a short-term

targeted inhibition in the early phase, e.g. by administration of neutralizing monoclonal antibodies with a very short half-life, may represent a further option [135-137].

2.4 The Complement system

The complement system, a protein cascade involved in immune responses, consists of around 40 fluid-phase and cell membrane-associated proteins[108]. It appears to represent the ‘‘key’’ mediator of innate immune responses after trauma [138-141]. The activation products of the cascade contribute to the production of other inflammatory mediators, and can therefore promote tissue injury at sites of inflammation [83]. Once activated through one of three established pathways, complement plays a critical role in the elimination of invading pathogens by opsonisation for phagocytosis (C3b, C4b), chemotaxis of leukocytes (C3a, C5a), and by direct lysis of pathogens through the membrane attack complex (MAC, C5b-9).[140, 142-144]. C1q is an essential component of the classic pathway, whereas C4 is essential to both the classic and lectin complement pathways. The classic pathway is initiated when C1q interacts with natural antibody bound to neoepitopes present on injured tissue, whereas the lectin pathway is initiated by a complex formed by mannose-binding lectin (MBL), immunoglobulins and MBL-associated serine proteases (MBL-MASP) [145] (**Fig. 3**). Both activated C1 complex and MBL-MASP complex may cleave C4 to C4a, C4b, and C4c. C4b and C2 react to form C4b2a, a C3 convertase that cleaves and activates C3, the convergence point for all complement pathways [145]. The alternative pathway requires the spontaneous breakdown of C3 in serum and factor B to assemble a C3 convertase Complement (**Fig.3**).

C3 is a key component in the activation of the complement system. The C3 precursor is a 185kD protein that is cleaved into an alpha (120kD) and a beta chain (75kD) linked by a disulfide bond. This mature C3 is further cleaved by C3 convertase to release C3a and C3b [146]. The anaphylatoxins C3a and C5a are potent chemo attractants for phagocytes and polymorphonuclear leukocytes (PMN, ‘‘neutrophils’’), and recruit these immune cells to the site of injury [147]. C3a is as well a potent vasoconstrictor and has been shown to be a promoter of inflammatory cytokines in human disease processes as diverse as cerebral ischemia [148] and Alzheimer’s disease [149]. The anaphylatoxins further induce degranulation of mast cells, basophils and eosinophils and mediate the hepatic acute-phase response [140, 143, 144]. The binding of C3a or C5a to receptors on oligodendrocytes and astrocytes may affect demyelination and scar formation

following CNS injury. The activation of C5aR by C5a peptide fragments has shown to trigger apoptosis in cortical and hippocampal cultures [150] and the activation of C3a and C5a receptors on astrocytes has been suggested to regulating scar formation and glial cell activation/chemotaxis [151, 152].

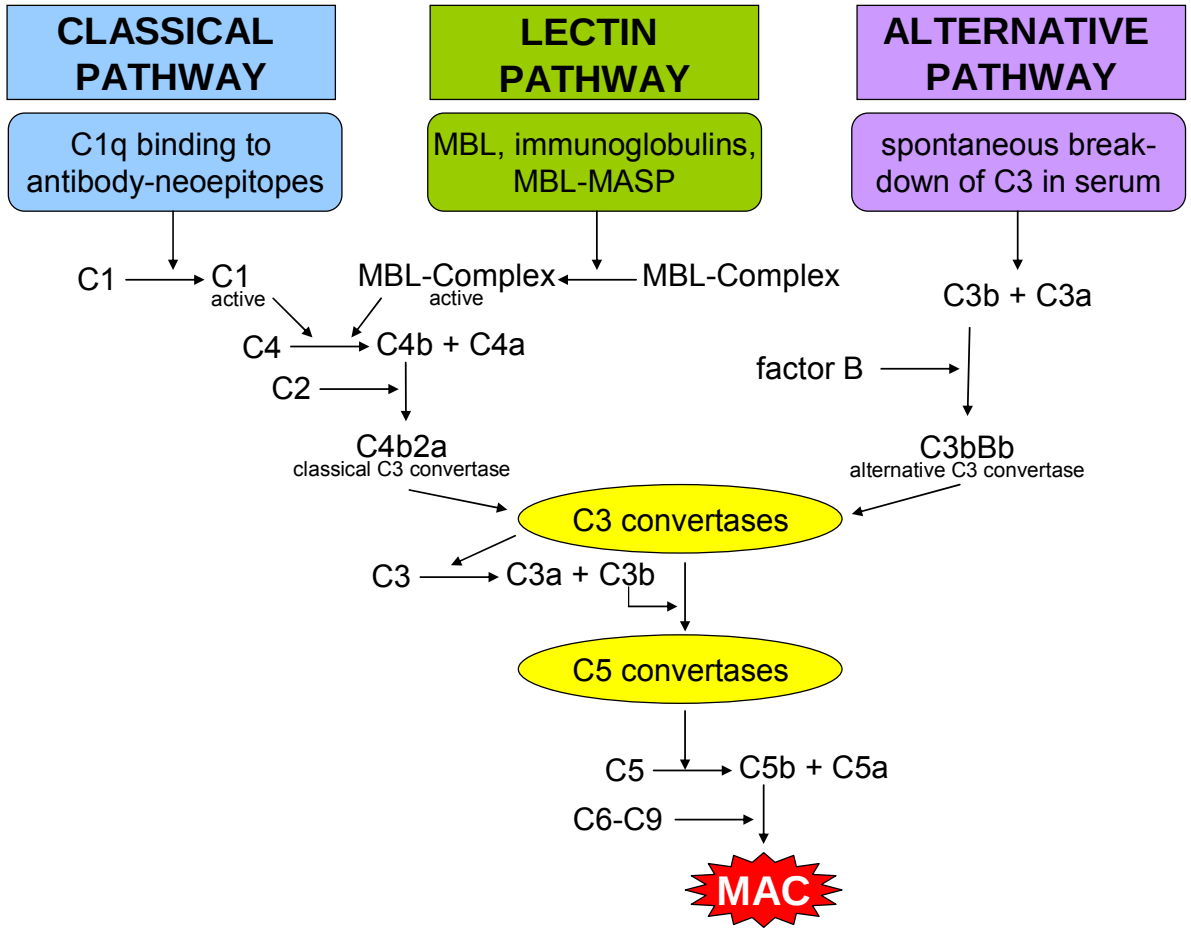

Figure 3. Activation of complement cascade via the classical, lectin or alternative pathways.
C1q is an essential component of the classic pathway, whereas C4 is essential to both the classic and lectin Complement pathways. The alternative pathway requires factor B to assemble a C3 convertase Complement.

Recently, it was discovered that C3a and C4a have direct antimicrobial activity, which provides an additional mechanism through which complement can kill microorganisms [153]. Finally, the generation of C5b by cleavage of C5 initiates the terminal complement pathway with MAC formation. The MAC forms through the self-association of C5b along with C6 to C9 and leads to the formation of a large membranolytic complex capable of lysing prokaryotic and eukaryotic cells. [140, 143, 144] by forming a pore in the phospholipids bilayer [154]. Clinical and experimental studies have demonstrated that complement activation occurs both locally, at the site of injury, as well as systemically after trauma.[34, 155, 156]. The systemic activation of the

complement cascade has been shown at the level of C3 in serum of trauma patients, and the extent of activation was correlated to the severity of injury [157, 158]

2.5 Complement expression in the central nervous system

Constitutive complement expression in the normal CNS is low, principally due to the tight separation between the vascular system and the intrathecal compartment by the BBB. However, a number of soluble complement components have been detected in the CSF under physiological conditions. Among these, complement proteins of the classical (C1q, C4) [159-161], and of the alternative activation pathway (C3, factor B) [161-165], as well as the terminal lytic pathway (C9) [166, 167] have been detected in normal CSF in constitutive low levels. Furthermore, complement components C1q, C3b and C3d are found in the vicinity of activated microglial, macrophage and astrocyte cells in the injured human brain [34]. C1q, C3, and other complement components contributed to brain edema and histopathology in models of ischemic and hemorrhagic brain injury [168, 169]. Increased cerebrospinal fluid C4a was associated with delayed ischemic neurological deterioration in patients with subarachnoid hemorrhage [170]. C4 mRNA and protein were induced in peri-infarct tissue after permanent focal stroke in mice [152] and neonatal mice deficient in C1q had reduced cerebral ischemic tissue damage compared with wild type (WT) [168]. C1q gene expression was increased in contused rat brain after experimental TBI [171]. Experimental closed head injury in wild-type C57BL/6 mice resulted in a systemic activation of the complement cascade, as determined by significantly elevated serum levels of the complement activation product C5a at all time-points assessed from 4 hours to 7 days [172].

Neurones, microglia, astrocytes and oligodendrocytes express complement proteins and receptors[33, 56, 173-177], where neurons are particularly susceptible to complement-mediated damage [83]. Moreover, glial cells are known to express receptors for C3a and C5a, and therefore are likely to take part in the complement-mediated events following CNS injury [178, 179]. Several studies have demonstrated the deposition of C5b-9/MAC on neurons and oligodendrocytes after traumatic CNS injury [180, 181]. Furthermore, neutrophil infiltration and an accumulation of C3 in cortical and hippocampal brain sections have been shown after a moderate Fluid percussion injury (FPI) in rats [37].

Proteins of the complement system are thought to originate mostly from the liver; however, previous studies have reported the expression or production of some

complement proteins by other cell types from the CNS [173, 182] and the immune system [183, 184].

The source of complement in normal CSF is either derived from passive leakage around the circumventricular organs, as demonstrated for C9[166] , or from intracerebral synthesis by resident cells of the brain[165, 173, 175, 176, 185]. The expression of complement proteins is prominently detected in the CNS 24 hours after TBI [145] [186].

A recent study from Nguyen et al. using a spinal cord injury (SCI) model in rates showed that cultured PMNs (in vitro) expressed mRNAs encoding for C1q, C3 and C4, where as Complement Proteins C1q and C3 were detected in less than 30% of cultured PMNs [187] 3 days after CSI about 70% of the PMNs exhibited C1q and C3 which shows that PMNs might contribute to the elevated levels of Complement in CNS after trauma[187].

2.6 Strategies for therapeutic inhibition of complement activation

C1 inhibitor has been shown to be therapeutically useful in a variety of animal models of inflammatory diseases, including gram-negative bacterial sepsis and endotoxin shock, suppression of hyperacute transplant rejection, and treatment of a variety of ischemia-reperfusion injuries (heart, intestine, skeletal muscle, liver, brain)[188]. In Humans, the use of C1 inhibitor has shown promising results in ischemia-reperfusion injury where a randomized double-blind study of 80 similar patients compared C1 inhibitor treatment with a control group among patients with acute ST-elevation myocardial infarction who underwent emergency coronary artery bypass surgery, revealed reduced cardiac troponin I levels in the treated group, as well as significant improvement in mean arterial pressure, cardiac index, stroke volume and a variety of other clinical criteria [189].

You et al showed recently that genetic inhibition of C4 in mice leaded to a reduction in post-injury motor deficits and a decreased brain tissue damage in a controlled cortical impact suggested [145].

Several early complement inhibitors have been investigated in experimental TBI models interfering with the cascade at the central level of the C3 convertases, where the three activation pathways merge [36, 190-194]. In a closed head TBI model, [192] reported that astrocyte overexpression of soluble complement receptor protein y (sCrry), a mouse-specific C3 convertase inhibitor, reduced posttraumatic blood– brain barrier damage and improved neurologic severity scores (including motor function, alertness, and physiologic behavior). Subsequently, we reported that systemic administration of

chimeric immunoglobulin G1-Crry (Crry-Ig) (**Fig. 4**) fusion protein also improved neurologic severity scores, and reduced hippocampal CA3/CA4 cell loss[191]. Other approaches were designed to inhibit the main inflammatory mediators of the complement cascade, such as the anaphylatoxin C5a [33, 186, 195, 196].

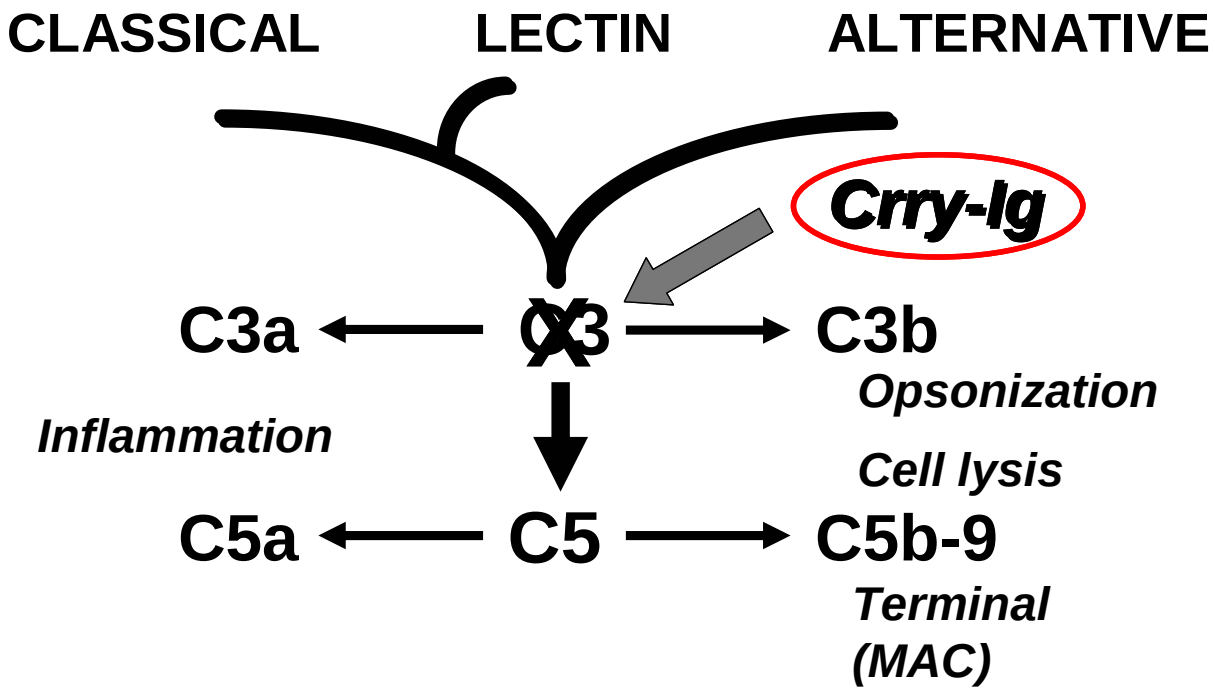

Figure 4. Simplified schematic of Complement inhibition in mice using Crry-Ig. Systemic administration of Crry-Ig after TBI showed improvement of the neurological outcome of mice.

Post-TBI administration of progesterone had reduced the expression of both the C3a and C3b fragments of factor C3 without influencing its overall expression, suggesting an effect on C3 convertase [197] and increased CD55 gene expression at 48h post TBI [198]. A reduction in C3b [3] can inhibit further activation of the complement system and the resultant amplification of the inflammatory process.

An immunemodulatory molecule, Vaccinia virus complement control protein (VCP), inhibit both classical and alternative pathways of the complement system and in so doing prevents cell death and inflammation [199]. Administration of VCP after moderate FPI showed a protective effect [200]. A recent study showed that VCP administration significantly influenced sensorimotor function recovery, but did not significantly improve the cognitive outcome after severe head trauma in Wistar rats. [201].

Some other studies have shown some controversy results showing the potentially protective role of complement. A previous study has shown that neural progenitor cells express receptors for complement anaphylatoxins C3a and C5a, and has suggested that complement promotes neurogenesis because C3 (-/-) deficient mice and mice lacking C3aR or mice treated with a C3aR antagonist had impaired ischemia-induced neurogenesis [202]. C5 deficient mice have shown increased intracerebral hemorrhage, worse neurological deficit and brain edema in comparison to C5 sufficient mice [203]. A recent study showed that MBL gene deficiency increases susceptibility to TBI in mice in Controlled cortical impact (CCI) trauma, where mice deficient in MBL had increased (almost two-fold compared to wild type) CA3 neuronal degeneration 6h after CCI.[204]. As a result novel and highly specific reagents such as exclusive inhibition of the alternative activation pathway.[172, 205-207] are currently under investigation in experimental models of traumatic injuries.

2.7 Apoptosis/ programmed cell death (PCD)

Apoptosis, the PCD means in Greek: “The fall of leaves in the Wind” [208, 209], and is known as a naturally occurring process of cell suicide that plays a crucial role in the development and maintenance of multicellular organisms by eliminating superfluous or unwanted cells [210-214]. This occurs during development, normal cell turn-over, hormone induced tissue atrophy and pathological processes such as neurodegenerative diseases [215],leading to death of millions of cells everyday in our bodies. Cells undergoing apoptosis show characteristic morphological changes, including shrinkage of the cell and its nucleus, plasma membrane blebbing, chromatin condensation and DNA fragmentation [210, 213].

All mammalian cells appear to express the basic machinery that mediates apoptotic cell death (e.g. a family of cysteine proteases, termed caspases), but the initiation of apoptosis is carefully regulated. Signals diverse as viral infection, extracellular survival factors, cell interactions and hormones may act to either suppress or promote the activation of “death program” [214].

Accumulating evidence strongly suggest that apoptosis contribute in a variety of neurodegenerative and neuroinflammatory diseases [216-218]. Induction of cell death can be induced through two distinct mechanisms, which are differentiated by morphological characteristics and different patho-physiological pathways. On one hand, neurons can undergo necrotic cell death in an early phase after brain injury, due to

direct mechanical damage to membranes and cytoskeleton with consecutive disturbances in ionic homeostasis and energy metabolism due to decrease intracellular ATP-levels. On the other hand, neuronal apoptosis/PCD occurs as an active process involving protein synthesis of endogenous nucleases. Physiological apoptotic cell death is crucial tissue remodelling and development and it is characterized by maintenance for of the cellular membrane integrity, DNA fragmentation, chromatine condensation and cell shrinking with ultimative degradation of the cell into membranous vesicles. Apoptotic cell bodies are then rapidly phagocytozed by immunocompetent cells [210-213]. Distinct signalling pathways of PCD have been described as intrinsic and extrinsic pathways of apoptosis (**Fig. 5**) [213]. While the intrinsic pathway is characterized by mitochondrial release of cytochrome C (Cyt c) into the cytosol and consecutive activation of caspases which ultimately lead to apoptotic cell death [219], the extrinsic pathway is mediated by extracellular "death factors", such as FasL,TNF and other members of the TNF-family, IL-18 and granzymes which correspond to enzymes released by natural killer(NK) cells[220]. Ligand-binding to specific receptors expressed on cell surfaces ("death receptors") activates intracellular "death domains" which are capable of triggering a cascade of cysteine proteases (caspases;IL-1β-converting enzyme related enzymes) whose activity is essential for almost all pathways of apoptosis [213, 214, 221] .

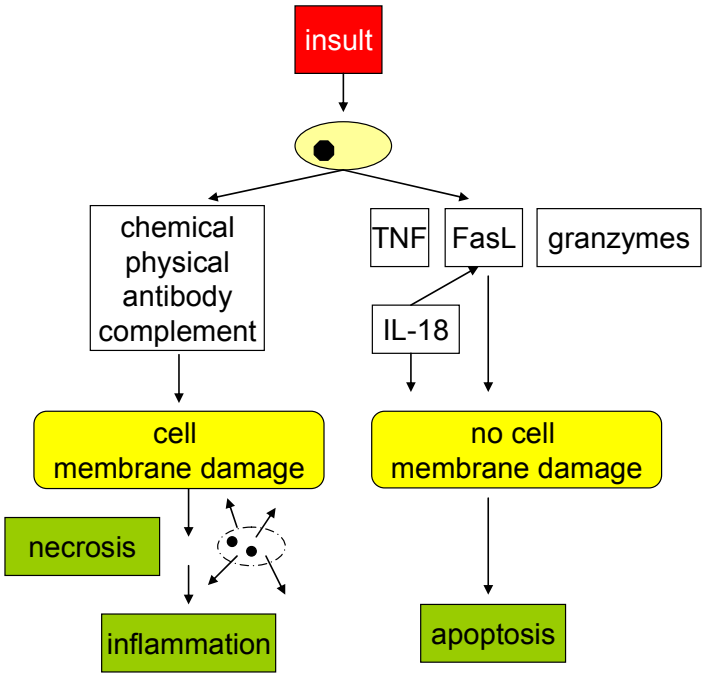

Figure 5. **Pathways of necrotic and programmed cell death (PCD).** Necrosis is initiated by chemical or physical injures or by antibody- or complement-mediated mechanisms. Mediators of apoptosis include Fas/FasL, TNF, IL-18 and granzymes. (See text for details)

2.8 The role of Apoptosis in Neurotrauma

In the past years, the mechanisms of neuronal cell death after TBI (**Fig. 6**) have received increased attention. It has been recognized that the process leading to brain parenchymal destruction and neuronal loss persist for a long period after trauma, based on studies in humans as well as in experimental brain injury models in rodents [94, 98, 99, 222, 223].

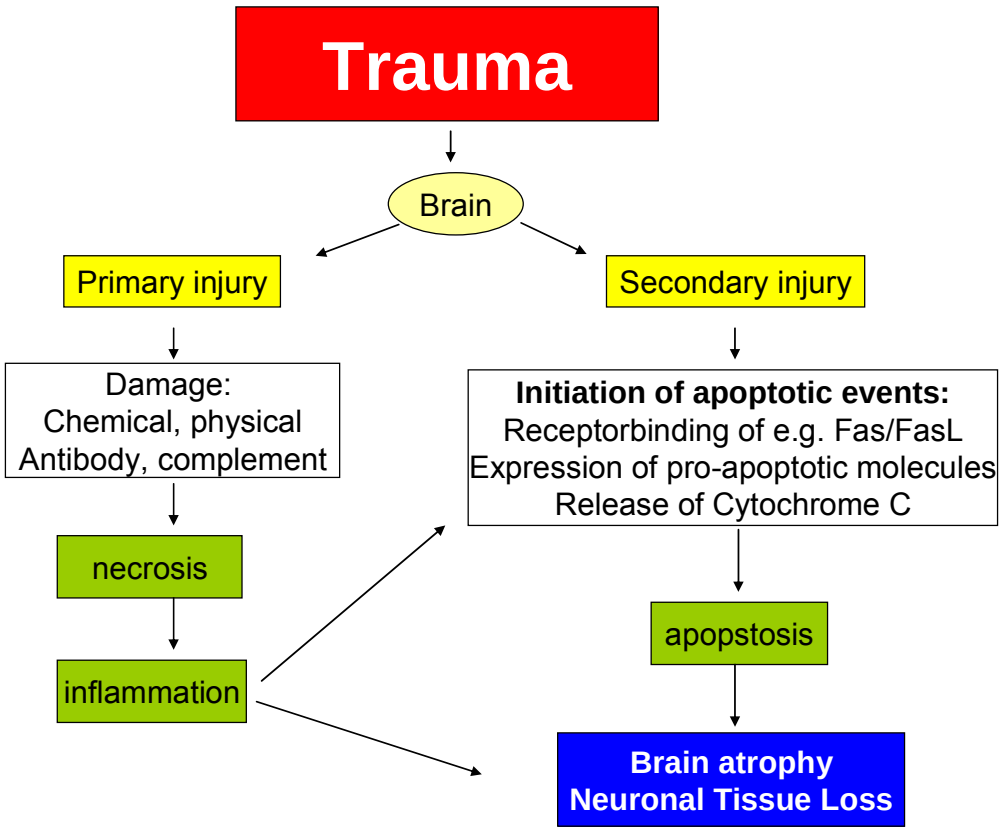

Figure 6. Proposed mechanisms of brain damage after traumatic brain injury. Primary brain injury due to the traumatic impact and resulting tissue damage leads to necrosis. Secondary brain injury is initiated by the inflammatory response and by intracellular activation of the apoptotic cascade, e.g. by ligand binding FasL to the Fas receptor. (See text for details)

Neuronal cell death is a very complex phenomenon possibly resulting from different events occurring simultaneously in response to trauma. Among these, the release of EAA, leading to excitotoxic cell death, and of reactive oxygen species (ROS) which induce mitochondrial dysfunction and consequent energy failure have been well recognized as causes of delayed neuronal death [224]. Evidence for apoptotic as well as necrotic cell death of neurons, astrocytes and EC has been reported in the brain of

rats subjected to fluid percussion injury and controlled cortical impact injury [93 , 225-227]. Within the cascade of apoptotic cell death, the involvement of the Fas/FasL-system, the tumour suppressor gene p53, caspases and anti-apoptotic factors of B-cell leukemia/lymphoma-2 (Bcl-2) family has been reported in various experimental brain injury models as well as in human TBI [94, 97-99, 101, 222, 223, 228-230]. Although apoptosis has been defined as a process of cell death independent from immunoactivation, apoptotic neuronal cell death has been observed at time-points which correspond to the elevation of intracerebral cytokine production after experimental head injury in mice[101, 105]. Whether or not inflammatory mediators can be considered as potential factors which mediate neuronal cell death after brain injury is still awaiting further experimental evidence. In a model of controlled cortical impact injury, Fas expression has been identified on astrocytes and neurons, while FasL increased also in microglial cells and both stainings overlapped with the regions displaying the highest distribution of TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) positive cells, as a sign of PCD-mediated DNA fragmentation [101].

Neurotoxin- or ischaemia-mediated apoptotic death was preceded by increased Bax mRNA and protein, and decreased expression of Bcl-2 in cells that are destined to die [231], while an Bcl-2 immunoreactivity was observed in neurons, glia and EC that survived focal ischemic injury[232]. Similarly, increased expression of Bcl-2 has been observed in neurons that survive the traumatic insult both in rat and in brain-injured humans [229, 233], while Bax was observed to translocate to the nucleus of apoptotic cells following experimental brain injury [227]. Increased expression of Bax and decreased expression of Bcl-XL have been reported at the site of injury in rat brain [234, 235]. An increased ratio of Bax/Bcl-2 has also been reported in rat cortex following TBI [236]. Alternatively, recent studies have suggested that decreases in intracellular Bcl-2 immunoreactivity, with little to no change in Bax proteins, in injured brain regions may precede cell death following experimental brain trauma [94, 236]. Transgenic mice overexpressing the human Bcl-2 protein exhibited significantly less neuronal loss in the injured cortex and hippocampus following experimental TBI, lending support to the idea that Bcl-2 may participate in the neuronal cell death following TBI [237, 238]. A pro-apoptotic member of the Bcl-2 family, Bid, has also been implicated in trauma-induced cell death in vivo—proteolysis of Bid preceding its translocation to the mitochondria has been demonstrated in the injured cortex [239].

Further downstream in the apoptotic cascade, the activation of poly-ADP-ribose-synthase, an enzyme mediating DNA repair, has been demonstrated early (30 min) in the injured cortex and has been shown to be cleaved later on (one week) and inhibit delayed repair of damaged DNA [240].

After experimental axonal injury, the alterations caused by tear-shearing injury of axonal membrane lead to Ca++ influx, cytoskeleton changes and mitochondrial swelling which are determined by the opening of the membrane permeability transition pores (PTP). The expression of Cyt c and caspase-3 have been associated to brain regions with greater mitochondrial damage, thus, converging the cascade of apoptotic cell death and energy failure [241]. Clinical studies revealed that sFasL, sFas, and caspase-3 activity are elevated in CSF of severely head injured patients [97-99]and that sFasL levels in CSF correlated significantly with severity on brain injury [97]. In addition, the prolonged intrathecal release of sFas correlated with the release of neuron-specific enolase as a marker of neuronal cell death, implying that activation of Fas-mediated PCD after neurotrauma may contribute to prolonged and delayed neuronal cell death [99]. In brain-injured children, Bcl-2 was elevated in CSF and in brain tissue samples from patients undergoing emergent decompressive craniectomies, suggesting that Bcl-2 may participate in the regulation of cell death in paediatric neurotrauma [230]. Further more the increase in Bcl-2 detected in surviving patients suggest a protective role for this ant-apoptotic protein after TBI [230]. Inflammation seems at least to modulate apoptosis indirectly, as pro-inflammatory cytokines have been shown to enhance the constitutive expression of Fas and FasL by cultured human astrocytes and astroglia [101, 242]. In addition, FasL-bearing astrocytes have been shown to undergo apoptosis in vitro and to induce apoptosis of co-cultured lymphocytes, suggesting that this mechanism of cell death may also occur after brain injury in neuronal cells [101, 242]. The exact mechanism of complement-mediated interaction with PCD remain unknown, thus linkage between innate immunity and apoptotic events need to be further investigated [243].

2.9 The BCL-2 Gene Family and its role in cell death regulation

Bcl-2 was identified originally as a novel pro-survival oncogene in B-cell lymphoma where, by chromosomal translocations, it was moved into juxtaposition with strong enhancer elements in the immunoglobulin heavy-chain locus [244, 245]. The result was the deregulation of the translocated Bcl-2 gene and the overproduction of Bcl-2 mRNA

and its encoded protein. On this consideration, Bcl-2 has been established to be a proto-oncogene that prolongs cell survival by inhibiting apoptosis. Subsequent studies have demonstrated that Bcl-2 can prevent or delay apoptosis induced by a large variety of stimuli in many cell types [246, 247], but the mechanism of its protective action has remained unclear. Soon after the discovery of Bcl-2, a family of proteins emerged that share with it differing degrees of homology and function [248]. Several hypotheses have been proposed to explain the anti-apoptotic function of Bcl-2. The Bcl-2 family of proteins may act as a regulator for Ca2+ homeostasis [249] or as an antioxidant [250]. In addition, Bcl-2 family of proteins plays a major role in regulating the intrinsic or mitochondrial apoptotic pathway, whereby Bax translocation from the cytosol to the mitochondria triggers the release of Cyt c to the cytosol [251-254]. Cyt c binds to apoptosis activating factor 1 (Apaf-1) in the cytosol (**Fig.7**) [255], which allows for the recruitment and subsequent activation of caspase 9, an initiator caspase, in this apoptosome complex [256]. Activation of caspase 9 in turn induces downstream activation of caspase 3, an effector caspase that when activated cleaves proteins such as poly ADP-ribosyl DNA polymerase (PARP), a DNA repair enzyme that when inactivated leads to apoptotic nuclear fragmentation [257].

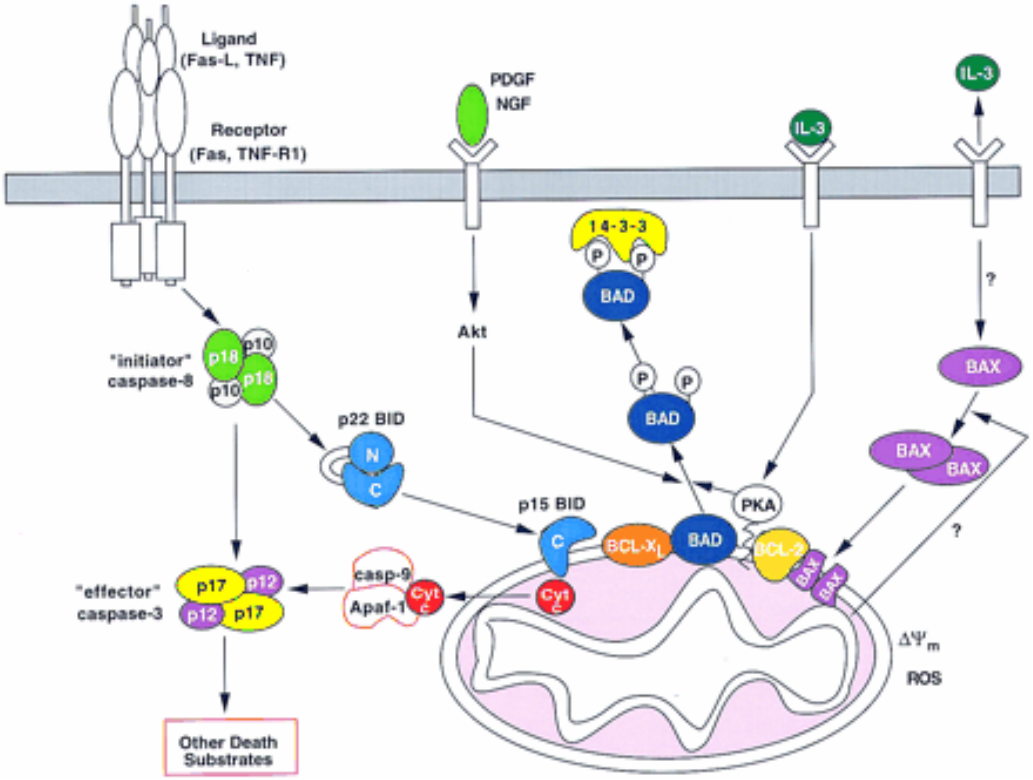

Figure 7. Model of apoptotic and survival signaling pathways involving the Bcl-2 members. [255] (Left) Activation of apoptotic pathways involving the TNFα/Fas cell surface receptor leads to activation of caspase-8. Caspase-8 cleaves cytosolic p22 BID generating a p15 carboxy-terminal fragment that translocates to the mitochondria resulting in the release of Cyt c. Released Cyt c activates Apaf-1, which in turn activates a downstream caspase program. (Right)A death stimulus (IL-3 deprivation) induces the translocation of Bax to the mitochondria where it is integral membrane and cross-linkable as a homodimer. (Center) Activation of the NGF or PDGF receptors mediates the activation of Akt, resulting in the phosphorylation of BAD at Ser-136. Activation of the IL-3 receptor mediates the activation of the mitochondrial-based protein kinase (PKA) holoenzyme, resulting in the phosphorylation of BAD at Ser-112. Phosphorylated BAD is sequestered to the cytosol by the phosphoserine-binding protein 14-3-3.

Bcl-2 may prevent the release of the mitochondria activators of the cytosolic caspases, which eventually mediates apoptosis-specific intracellular proteolysis [246]. The initiation and maintenance of Bax-dependent apoptosis has been studied extensively and is regulated through a complex system of protein modifications of Bcl-2 family members and their interacting partners. Also, Bcl-2 might act by modulating the collapse of the mitochondrial transmembrane potential that occurs during apoptosis [247]. In addition, the initiation of Bcl-2 family-dependent apoptosis depends not only on the relative expression levels of its pro- versus anti-apoptotic members but also on death stimulus and its severity [248, 255] (**Fig. 8**).

By various biochemical, genetic, and molecular techniques, numerous homologous of the Bcl-2 have been identified in vertebrates, forming a Bcl-2 family of proteins. This family includes proteins, which can promote either cell survival, such as Bcl-2, Bcl-XL,

Mcl-1, A1, Bcl-W[258] or cell death, like Bax, Bak, Bcl-XS, Bok [246]. The relative amounts or equilibrium between these pro- and anti-apoptotic proteins influence the susceptibility of cells to a death signal.

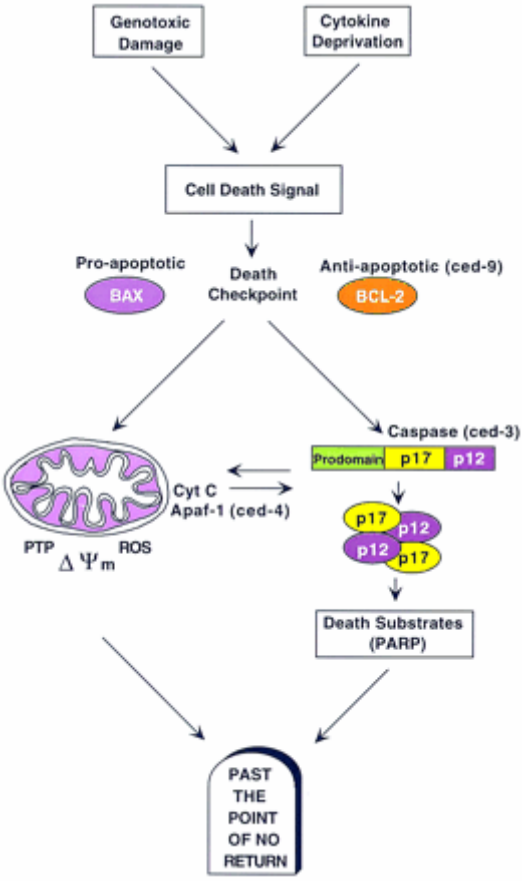

Figure 8. Schematic model of mammalian cell death pathway. [255]. A major checkpoint in the common portion of this pathway is the ratio of pro-apoptotic (Bax) to anti-apoptotic (Bcl-2) members. Downstream of this checkpoint are two major execution programs: the caspase pathway and mitochondria dysfunction. Mitochondrial dysfunction includes a change in the mitochondrial membrane potential ($\Delta\psi m$), production of ROS, opening of the PTP, and the release of the intermembrane space protein, Cyt c. Released Cyt c activates Apaf-1, which in turn activates a downstream caspase program. Activated caspases can also affect the function of mitochondria. Caspases could be activated through Apaf-1/ Cyt c or directly by activation of cell surface death receptors. Caspases (e.g., caspase-3) are activated by two cleavage events that occur between the prodomain and the large subunit (p17) and between the large subunit and the small subunit (p12). The activated caspase, composed of two large and two small subunits, cleaves death substrates (e.g., PARP), which ultimately leads to cell death.

Unrelated to their role in apoptosis, all Bcl-2 family members are characterized by containing at least one of four conserved Bcl-2 homology (BH) domains, designated BH1-BH4, which correspond to α-helical segments [258]. In general, the anti-apoptotic members show sequence conservation in all four domains (**Fig. 9**), while the pro-apoptotic molecules are characterized by loosing the sequence conservation of the first α-helical segment, BH4[259]. The BH3 domain is presumed as a critical death domain

in the pro-apoptotic members. This concept is supported by "BH3-domain-only" members (**Fig. 9**), who show sequence homology only within the BH3 domain and to date, are all pro-apoptotic [260]. The Bcl-2 protein is a membrane protein that is localized to the outer mitochondria membrane, endoplasmic reticulum membrane, and nuclear envelope, whereby it's NH2-terminal is facing the cytosol. As many other members of its family, Bcl-2 has a hydrophobic domain at COOH-terminal (TM domain, see (**Fig. 9**)) that allows the insertion of the protein into the cytosolic face of the intracellular membranes [261]; this intracellular localization is important for their function.

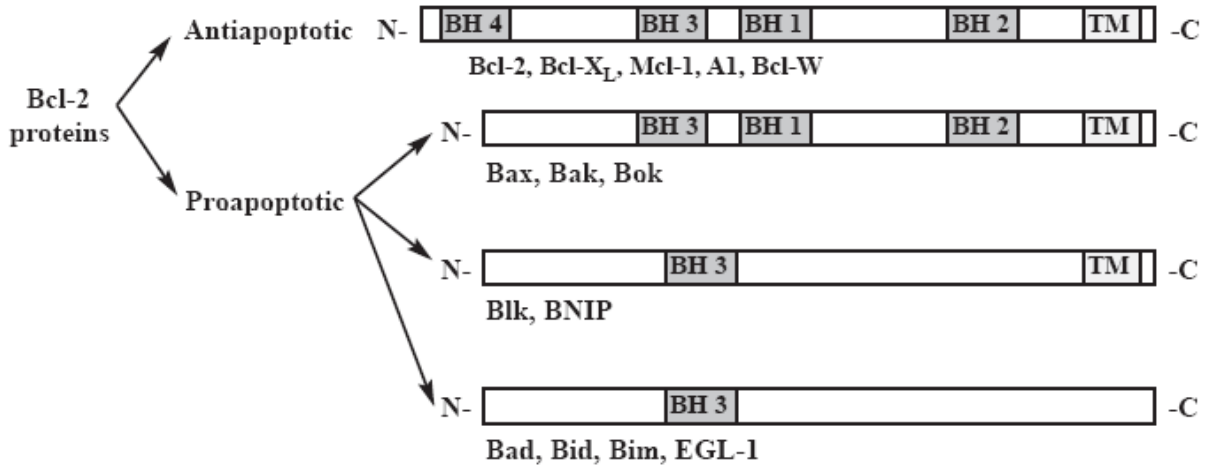

Figure 9. Schematic representation of the structural features of anti-apoptotic and pro-apoptotic Bcl-2 proteins[259]**.** The structures of proteins are presented in linear forms, BH1-4 indicate the homologue domains and TM the carboxy-terminal hydrophobic transmembrane domain. The Bcl-2 proteins include: anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl1, A1, Bcl-W) with all four BH and the TM domains, pro-apoptotic proteins (Bax, Bak, Bok) having TM, and BH13 domains but without BH4 domain, and pro-apoptotic ligands possessing only the BH3 domain with (Bik, BNIP) or without (Bad, Bid, Bim, EGL-1) TM.

An important feature of the members of Bcl-2 family is their ability to form homo- as well as heterodimers, suggesting neutralizing competition between these proteins. Thus, Bcl-2 (a 239 aminoacids protein, 26 kDa) forms heterodimers with Bax, a proapoptotic protein with ~ 21% aminoacid identity with Bcl-2 [258]. In addition to Bax, several other genes have been reported to encode proteins having sequence homology with Bcl-2 and capacity to form heterodimers with it. Among these, Bcl-X is able to generate two proteins through an alternative splicing mechanism: Bcl-XL (longer form) and Bcl-XS (shorter form). Bcl-XL is a 241 aminoacids protein that suppresses the cell death, having 43% sequence identity with Bcl-2, while Bcl-XS, in which a 63-aa region (aa 126-188) found in Bcl-XL is missing, functions as a proapoptotic protein [258]. Another Bcl-2

homologous is Mcl-1, which shares ~ 35% sequence identity with Bcl-2 over a region of ~ 140-aa [262]. Until now, it was not clearly demonstrated whether Mcl-1 is able to interact with Bcl-2, or functions in the regulation of cell death.

Regarding the intracellular localization of Bcl-2 family members, in the absence of a death signal, pro- and anti-apoptotic Bcl-2 proteins are localized to distinct intracellular compartments, providing important clues for their function [263]. Anti-apoptotic members are initially integral membrane proteins found especially in the mitochondria, endoplasmic reticulum, and nuclear membranes [264]. The large majority of the pro-apoptotic proteins are localized to the cytosol, but following a death signal, it appears that they undergo a conformational change that enables them to target and integrate into mitochondria outer membrane and to function as pro-apoptotic proteins [251, 265-267].

Bcl-2 is the prototypical member of the Bcl-2 Family [268]. Bcl-2 mRNA and protein are present at relatively high levels in the developing nervous system and decline significantly in the postnatal brain [269, 270]. Bcl-2 expression is maintained at relatively high levels in sensory and sympathetic neurons in the adult peripheral nervous system [270, 271]. The role of Bcl-2 in neuronal PCD has been examined using several experimental approaches. Trophic factor withdrawal-induced death of a variety of neuronal cell lines and primary neuron populations, an vitro model of target-dependent programmed cell death, is inhibited by Bcl-2 overexpression [272, 273]. Similarly, neuronal overexpression of Bcl-2 in transgenic mice increases the number of neurones in many brain regions by inhibiting naturally occurring neuronal death [273, 274]. These overexpression studies, however, only indicate a potential role for endogenous Bcl-2 in regulating PCD since other anti-apoptotic family members may play a more significant role in vivo. [275]. Targeted disruption of the Bcl-2 gene has indeed revealed selective effects of endogenous Bcl-2 expression on different neuronal populations.

Despite high levels of Bcl-2 mRNA and protein expression in both neural precursor cells and in post-mitotic neurons in the embryonic brain, Bcl-2-deficient embryos show relatively normal nervous development and no significant increase in neuronal programmed cell death[275]. Bcl-2 deficient mice exhibit a profound loss of motor neurons, sympathetic neurons and sensory neurons during early postnatal life [276-278]. Unlike neurons in the brain, these peripheral neuronal populations normally exhibit significant baseline Bcl-2 expression during the neonatal period [270]. The death of these neurons in Bcl-2 deficient mice occurs after the normal peak of PCD in these

populations, suggesting that Bcl-2 may play a role in maintenance of their survival rather than in regulating their PCD per se. [275].

The pro-apoptotic molecule Bax was originally identified as a binding partner for Bcl-2 [279]. Bax is expressed in both the embryonic and adult brain and can heterodimerize with Bcl-2, Bcl-XL, Mcl-1 and A1 [262, 279-281]. Current data suggest that Bax regulates Cyt c release from mitochondria perhaps via formation of the mitochondrial transition pore [254, 282]. Anti-apoptotic members of the Bcl-2 family may heterodimerize or hetero-oligomerize with Bax to inhibit Bax function. Targeted gene disruption of Bax has demonstrated an important role for Bax in triggering neuronal programmed cell death.

In the developing nervous system, PCD of synapse-bearing neurons during a period of competition for target-derived neurotrophic support helps determine neuronal cell members. Bax-deficient mice exhibit markedly decreased neuronal PCD in a variety of sites including brainstem, cerebellum, dorsal root ganglia, hippocampus, and spinal cord, with resultant increased neuron numbers in these areas[283, 284]. This phenomenon is recapitulated in vitro in neonatal sympathetic neuron cultures. Following NGF withdrawal, wild-type sympathetic neurons exhibit redistribution of Cyt c from the mitochondria to the cytosol and undergo fairly rapid apoptotic death[283, 285]. In contrast, Bax deficient sympathetic neurons do not show increased cytosolic Cyt c and survive for weeks after NGF withdrawal [285]. Bax-deficient neurons under these conditions exhibit reduced soma size and axon diameter. Neuronal atrophy is also observed in vivo in some populations of Bax-deficient neurons, indicating that neurons “rescued” from PCD may not be completely normal [283, 284]. Interestingly, in certain models of trophic factor withdrawal-induced neuronal death, Bax may not play a critical role. For example, in NGF-deprived trigeminal neurons, Bax-deficiency delays, but does not prevent, cell death [286]. Also, in cultured chick sensory and ciliary neurons, Bax over-expression may have a pro-survival action following trophic factor withdrawal [287]. Bax may also play a role in regulating activity dependent neuronal apoptosis. Cerebellar granule neurons (CGNs) undergo apoptosis when cultured in non-depolarizing, serum-free media, and Bax-deficient CGNs are protected from this apoptotic stimulus [288, 289].

Targeted disruptions of both Bax and Bcl-X revealed important roles for these Bcl-2 family members in neuronal programmed cell death. To examine the potential interaction of Bax and Bcl-XL in neuronal cell death regulation, Bax/Bcl-XL deficient

embryos were generated. Bax, Bcl-XL dual-deficient embryos showed complete protection from Bcl-XL deficiency-induced neuronal apoptosis [290]. The neuroprotective effect of Bax deficiency on Bcl-XL -deficient cells was also observed in vitro in primary telencephalic neuronal cell cultures [290]. Bax deficiency did not, however, rescue Bcl-XL-deficient mice from embryonic lethality, indication that Bcl-XL has both Bax-dependent and Bax-independent actions on different cell types [290].

Although Bax deficiency inhibits PCD in many neuronal populations, Bax-dependent death pathways also contribute significantly to nervous system morphogenesis. Like Bax-deficient mice, mice with targeted gene disruptions of caspase-9, apaf-1 and caspase-3 exhibit markedly reduced PCD. However, unlike Bax-deficient mice, a significant number of embryos with caspase-9, apaf-1 and caspase-3 gene disruptions also possess gross structural brain abnormalities, including neural precursor cell hyperplasia and forebrain exencephaly [291-294].

This conclusion requires caution, however, for several reasons. First, the neurodevelopemental effects of caspase-9, apaf-1 and caspase-3 gene disruption are incompletely penetrant and strain-dependent. For example, caspase-3 deficiency causes perinatal lethality and severe neurodevelopmental pathology in 129x1/SvJ mice. In contrast, the same gene disruption in C57BL/6J mice leads to minimal neuropathological abnormalities[295]. This result indicates that direct comparisons between gene disruptions on different or mixed genetic backgrounds are problematic. Second, Bcl-2 family members have overlapping actions, thereby raising the possibility that changes in the expression of other family members may compensate for Bax deficiency [275].

3. Specific aims of this project

3.1 Experimental Study

Analysis of the pro and anti-apoptotic proteins (Bcl-2 and Bax) regulation following experimental TBI in mice (wild type, vehicle injected mice and Crry-Ig injected mice) using Western Blot.

3.2 Experimental Study

Assessment of the intracerebral expression of the Bcl-2 and Bax genes. A semi-quantitative two-step real-time RT-PCR using murine-specific primers was utilized.

3.3 Experimental Study

Revealing of the Neuronal morphology, integrity and microarchitecture using a monoclonal antibody against the murine neuronal cell-marker NeuN.

3.4 Experimental study

Detection of Intracranial cell death in brain cells following experimental TBI in mice by the detection of DNA fragmentation with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) technique.

4. Materials and Methods

4.1 Chemicals

Chemicals	Company
Acrylamid/Bisacrylamid (37, 5: 1) (Rotiphorese Gel)	Roth
Ammoniumpersulfat (APS)	Roth
Aprotinin	Sigma
BCA Protein Assay Kit	Pierce
β-Mercaptoethanol	Merck
Bromphenol blue	Biorad
Coomassie Blue	Roth
Deionized water	Biochrom AG
DMSO (Dimethyl sulfoxide)	Roth
ECL solution (developing substrate)	Amersham
Ethanol	Merck
Glycerol	Roth
Glycine	Serva
Igepal CA-630	Sigma
Isoflurane	Forene
Kaleidoscope Polypeptide Standards	Biorad
Leupeptin	Roche
Methanol	J.T.Baker
Non-Fat Dry Milk	Biorad
PBS (Phosphate-buffered saline)	Biochrom AG
Pepstatin	Roche
PMSF(Phenylmethanesulfonylfluoride)	Sigma
Ponceau S	Sigma
Precision Plus Protein Standards	Biorad
Prestained SDS-Page Standard (Broad Range)	Biorad
SDS(Sodium Docecylsulfate)	Sigma
Sodium chloride	Roth
Sodium hydroxide	Roth
TEMED	Roth
Tris (Base)	Roth
Tris-HCl	Roth
Tween-20	Sigma

Table 2 shows all the chemicals used during the experiments and their producing company.

4.2 Other materials

Materials	Company
Blue und Red Cap Tubes (50 ml)	Falcon
Cryo test tubes	Falcon
Disposables for cell culture (96 wells)	Nunc
Film cassette	Kodak
Film developing machine	Kodak
Filter paper 3MM	Schleicher und Schuell
Heparin syringe	Becton Dickinson
Hyper film	Amersham
Micro filter	Nalgene
Microliter syringe	Hamilton
nitrocellulose (Protran BA 83)	Schleicher und Schuell
PP-Tube	Greiner bio-one
Professional wipes	Kimwipes
Safe lock tubes	Eppendorf
Scalpel	Feather
Serological pipette	Falcon
Transparency film	Tartan

Table 3 Shows all the other materials used during the experiments and their producing company.

4.3 Laboratory equipment

Laboratory equipment	Company
balance	Scaltec
Centrifuge (2k15)	Sigma
Centrifuge with JA25.25 rotor	Beckman
Dispersing tool (T18 basic)	IKA works
Dri Block (DB-2D)	Techne Dri-Block
Electronic pipette	Neo Accupette
Freezer (-20°C)	Liebherr
Freezer (-80°C)	Bioblock Scientific
Ice machine	Ziegra
Incubator	Memmert
Macro pipette controller	Brand
Magnitic stirrer	Hanna Instruments
Micro plate reader	Biorad
Microbiological safe working bench	Clean air
Mini Electrophoresis model	Biorad
Mini Transfer model	Biorad
Mixer (Vortex Genie 2)	Scientific Instuments
Multi pipette	Eppendorf
PH meter	Fischer Scientific
Pipette	Eppendorf
Power supply (Powe Pac)	Biorad
Precision balance	Denver
Refrigerator (4°C)	Bosch
Scanner	Hewlett Packard
Shaker	Edmid Bühler
Table centrifuge (Micro 7)	Fischer Scientific
Water deionizing machine	Millipore
Waterbath	Memmert

Table 4 shows all the laboratory equipments which were used during the experiments and their producing company

4.4 Antibodies

4.4.1 Primary antibody

A dilution series, in 3% or 5% (depending on the antibody) non-fat dry milk in PBS (phosphate-buffered saline) -Tween (0.05%) was performed for all antibodies used in this project.

To detect the anti-apoptotic oncoprotein Bcl-2, a monoclonal mouse anti human Bcl-2 (Santa Cruz Biotech. Inc, Santa Cruz, CA, U.S.A) was used at a dilution of 1:500 in 3% milk and incubated for 2:30h. The pro-apoptotic protein Bax was detected using a polyclonal rabbit anti human Bax-α (Santa Cruz Biotech. Inc, Santa Cruz, CA, U.S.A) at a dilution of 1:200 in 5% milk and incubated for 1h.To detect the cytoskeletal protein β-

Actin a monoclonal anti-mouse antibody (Sigma, Saint Louis, Missouri, USA) was utilised and diluted 1:5,000 in 5% milk and incubated for 1h.

4.5 Animals

4.5.1 Breeding conditions

All animals had been acclimatised several weeks before the experiments and were kept isolated from the external influences during the entire time course of the study in the Laboratory (Forschungseinrichtung für Experimentelle Medizin, Berlin, Germany).

They were housed in groups of 10 per cage and separated into individual cages once they reach 25 g. They were bred in a specific pathogen-free (SPF) environment, kept under standard conditions of temperature(21°C), humidity(60%), light and dark cycles(12h:12h). They were fed with food and water ad libitum (Crude proteins 21%, Crude fat 3,8%, Crude fibre 4,4%, Crude ashes 6,7%) for the first 6 weeks of life then ad libitum (Crude proteins 20%, Crude fat 3,5%, Crude fibre 4,9%, Crude ashes 6,7%) for the following weeks. The experiments were performed in compliance with the standards of the Federation of European Animal Science Association (FEASA) and have been approved by the institutional animal care committee (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin, Germany, No. G0099/03).

4.5.2 Wild type mice

The C57BL mouse was originally derived by Little in 1921. Today, it is one of the most widely used and most popular strains in animal research.

C57BL/6 is classified as an inbred, which results from a crossing of no less than 20 consecutive generations of brother-sister matings. This allows the offspring to possess both genetic and phenotypic uniformity. As with most inbred strains, reproductive performance is lowered, along with the general size of the mouse and its overall lifespan, in comparison to its outbred counter part.

All mice (Jackson Laboratory, Bar harbor, ME, USA) (total n=54) were males of age 8-16 weeks, with an average weight of 28-32 g. They were randomized into 6 cohorts; group 1: normal mice (negative control) without trauma, group 2: sham-operated mice without trauma, group 3: trauma, group 4: trauma with posttraumatic intra peritoneal (i.p.) injection of 0.4ml PBS (vehicle) t=1h, t= 4 and t=24, group 5: trauma with Crry-Ig

4.5.3 Negative control (nil) group

For the assessment of the baseline characteristics in normal (untreated) animals, these mice (n=3) were kept under identical conditions as the sham-operated, head injured, injected and knockout animals, but no experimental procedure was performed.

4.5.4 Sham-operated control group

These mice (n=9) were kept under the same conditions as the other animals, underwent anaesthesia and scalp incision but no head injury or injection were performed. Sham-operated mice were sacrificed for brain tissue extraction and neurological assessment at t=4h, 24h, 7 days and 4weeks.

4.5.5 Trauma group

These mice (n=9) were kept under identical conditions as the other animals, underwent anaesthesia, scalp incision and trauma but were not injected at all. They were decapitated at time-points t=4h, 24h, 7 days and 4weeks.

4.5.6 Vehicle injected

This group of mice (n=13) were used as control group for the Crry-Ig and anti factor B injected mice. They were exposed to the same circumstances as these mice but received a posttraumatic i.p. injection of 0.4ml PBS (vehicle) t=1h, 24 and 7days. They were killed at time points corresponding to the other injected mice.

4.5.7 Crry-Ig injected mice

The C57BL/6 mice (n= 10) were exposed to trauma and then injected posttraumatic i.p with recombinant Crry-Ig. 1 mg Crry-Ig per mouse was chosen as standard dose, which is in titrated range used in previous studies on other murine models of inflammation [296-298]. The systemic (i.p) route of administration and the time window of injection was selected based on the following rationale: (a) In this model, the BBB is breached as early as 1 h after trauma, peaking at 4 h, and persisting up to 24 h [96, 299]. This allows a “time window of opportunity” for peripherally administrated compounds to reach the intrathecal compartment and exert pharmacological effects in the inflamed CNS, as previously shown for other pharmacological agents [117, 300, 301] ;(b) The post traumatic systemic injection within 1h after injury represents an approach with potential clinical implications; (c) A previous study using a different model of murine

neuroinflammation has determined that i.p. route of administration of Crry-Ig is feasible and enables Crry-Ig-mediated anti-inflammatory effects in brain [296].In order to induce a continuing complement inhibition during the acute inflammatory phase in the first days, a protocol of repeated i.p. injections of 1mg Crry-Ig in 4ml PBS was defined for t=1h and t=24h after the induced trauma. The half-life of this compound was determined at $t_{1/2}$=40h after systemic injection in the mouse [297] The mice were sacrificed at time-points t=4h, 24h, 7 days and 4weeks.

4.6 Model of closed head injury (CHI) in mice

We use a CHI model which was developed at first in Rat [302] and later established in mice [299] in which a new mechanical device was build, with a falling weight adjusted for the size and weight of the mouse. The mouse model has been used by Esther Shohami's group in various studies[120, 303-308]. Mice were anaesthetised using isoflurane for about 1 min., the skin covering there skull was longitudinal incised and retracted as to expose the skull. The head was manually fixed at the bottom of the impact device (B) (**Fig. 10**) and Focal trauma was induced to the left hemisphere, 2 mm lateral to the midline in the midcoronal plane, using an electric weight-drop device with a metal rod (A) of 333 g, falling from a height of 2 cm. The chosen height is maintained by an electric lock (D) controlled by a foot-touch switch (E). The impacting rod was supplemented with a silicone tip(C) of 3 mm diameter at its end, in order to avoid penetrating skull fractures.

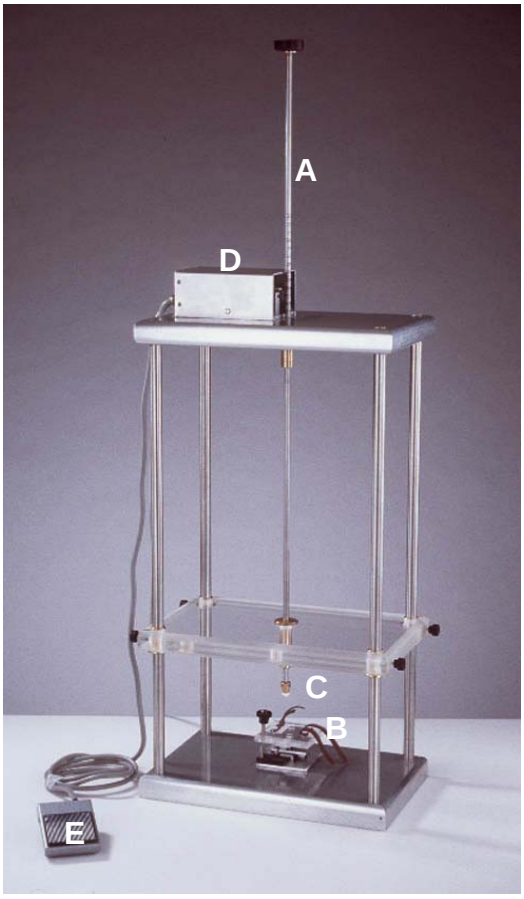

Figure 10. Schematic diagram of the electric weight-drop device.(Shohami et al.) metal rod (A); impact device (B); silicone tip(C); electric lock (D); foot-touch switch (E).

The mice received supporting oxygenation with 100% O_2 after trauma until they awakened, usually within 2-3 min and then bought to their cages, with food and water ad libitum. The animals were killed (decapitated) in isoflurane anaesthesia 4 hours, 24 hours, 7 days or 4 weeks after trauma and the brains were frozen in liquid nitrogen and stored at -80^{o}C until analysed. Blood was withdrawn using a 1ml heparin syringe inserted in the mouse's heart. The blood is centrifuged at a speed of 13000 Xg for 20min and then serum is separated, aliquoted, immediately frozen in liquid nitrogen and stored at –80°C till later analysis.

4.7 Neurological scores

To assess the posttraumatic neurological impairment, a 10-parameter Neurological Severity Score (NSS) has been previously developed and validated in Esther Shohami's laboratory in Jerusalem [96, 304]. The original score was 25 -point, which was previously used for assessment of neurological impairment in the same model

system[299]. It was reduced to 10 “essential” parameters which assess motor function, alertness, balancing and physiological behavior of each mouse.

One point is awarded for failing to perform a particular task and zero points for succeeding (**Table 5**). The NSS was assessed at time-points1 hour, 4 hours, 24 hours and 7 days after trauma. Evaluation of task performance was performed by two investigators who were blinded about the animal groups. A maximal NSS of 10 points indicates severe neurological dysfunction, with failure of all tasks, whereas a score of zero is achieved by healthy uninjured mice. When a mouse was dead, it was excluded from the NSS evaluation.

Task	Description	Points Failure / success
Exit circle	Ability and initiative to exit a circle of 30 cm diameter (time limit: 3 minutes)	1 / 0
Mono-/Hemiparesis	Paresis of upper and/or lower limb of the contralateral side	1 / 0
Straight walk	Alterness, initiative, and motor ability to walk straight, once the mouse is put on the floor	1 / 0
Startle reflex	Innate reflex: the mouse will bounce in response to a loud hand clap	1 / 0
Seeking behavior	Physiological behavior as a sign of interest in the environment	1 / 0
Beam balancing	Ability to balance on a beam of 7mm width for at least 10 seconds	1 / 0
Round stick balancing	Ability to balance on a beam of 5mm width for at least 10 seconds	1 / 0
Beam walk: 3 cm	Ability to cross a 30 cm long beam of 3 cm width	1 / 0
Beam walk: 2 cm	Same task, increased difficulty on a 2 cm wide beam	1 / 0
Beam walk: 1 cm	Indem, increased difficulty on a 1 cm wide beam	1 / 0
MAXIMAL SCORE		10

Table 5. **Neurological Severity Score (NSS) for mice**

4.8 Western Blot

4.8.1 Homogenization (emulsification) of Mice's Brain

All steps are carried out on ice. The brains from Sham as well as trauma animals are cut into left and right hemisphere and placed in a 14ml test tube. 3ml lyses buffer (**Table 6**) is added to the test tube and the brain is dispersed on level 5, 3 X 10 seconds (Ultra Turrax T18, IKA). Then centrifuged at a speed of 13000 x g for 15min. in a Beckman Centrifuge, Rotor JA25.1. The Probes are aliquoted and stored in -20°C.

Chemical	amount	End concentration
Tris-HCl (1M pH 7,5)	100 µl	0.01M
NaCl (3M)	480 µl	0.144M
SDS (10%)	500 µl	0,5%
Nonidet P40	50 µl	0,5%
Aprotinin Tyrasylol	10 µl	10µg/ml
Leupeptin (1mg/ml)	100 µl	10µg/ml
Pepstatin (0.5mg/ml)	100 µl	5µg/ml
PMSF (100mM in DMSO)	100 µl	1mM
Deionized water	8,5 ml	

Table 6. Lysis buffer (10ml)

4.8.2 Protein quanitation BCA (bicinchoninic acid) Protein Assay Reagent:

A series of dilutions was prepared from 2 mg/ml BSA (stem solution) in Lysis buffer: 1500, 1000, 750, 500, 250, 125, 25 und 0 µg/ml BSA. The protein probes were diluted 1:10 in lysis buffer. The series of dilutions were pipetted as well as the unknown probe 3 times, each 10µl in a 96 well microplate. Reagent A (196µl/probe) and reagent B (4µl/probe) were mixed together then 200µl were added to each probe. The probes were incubated for 30min. in 37°C and then measured with spectrophotometer set to 550 nm. 60µg is divided by the given value as to calculate the amount of protein to be loaded into wells formed in the gel using a well-forming comb.

4.8.3 Discontinuous [1] SDS-Page

Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (Page) separate the denatured proteins by molecular weight [1].The proteins runs toward the positive pole through stacking (upper) gel to concentrate the large sample volume and then through separating (lower) gel. The molecular weight of unknown proteins can be determined with the help of blotting protein standards.

The Gels are prepared in a Mini-protean II electrophoresis cell (BIO RAD) where TEMED und APS are added directly before the solution is poured. At first the separating gel (**Table 9; Table 10**) is poured till 1 cm below the teeth of the comb and covered with deionized water. After polymerisation (30 minutes), the water is poured out, the area above the separating gel is dried with professional wipes (kimwipes) and stacking gel (**Table 11**) is poured.

Chemical	Amount
Tris-Base (0.64 M)	38.5 g
Tris-HCl(0.12 M)	9.3 g
SDS(0.2%)	1.0 g
adjust pH to	8.8
make to 500 ml with deionized water	

Table 7. Separating gel buffer

Chemical	Amount
Tris-Base(0.33 M)	19.7 g
SDS(0.2%)	1.0 g
adjust pH to	6.8
make to 500 ml with deionized water	

Table 8. Stacking gel buffer

Chemical	Amount
30% Acrylamid/Bis 37,5:1(2.6 % C)	4 ml
Separating gel buffer	5 ml
Deionized water	890 µl
TEMED	10 µl
10% ammonium persulfate	100 µl

Table 9. Separating gel 12% (10ml)

Chemical	Amount
30% Acrylamid/Bis 37,5:1(2.6 % C)	5 ml
Separating gel buffer	5 ml
Deionized water	-
TEMED	10 µl
10% ammonium persulfate	100 µl

Table 10 Separating gel 15% (10ml)

Chemical	Amount
30% Acrylamid/Bis 37.5:1(2.6 % C)	890 µl
Stacking gel buffer	2.5 ml
Deionized water	1.7 µl
TEMED	10 µl
10% ammonium persulfate(APS)	50 µl

Table 11 Stacking gel 5% (5ml)

A comb with 10 teeth is placed in the stacking gel, the gel is allowed to polymerize (30 minutes) then the comb is removed by pulling it straight up slowly and gently.

The samples are prepared from proteins samples (60μg) and lysis buffer. Probe buffer (**Table 12**) is added at a ratio 3:1 and heated at 95°C for 5 minutes as to denaturize the proteins. During this time the upper buffer chamber is assembled and the wells are rinsed with electrode buffer (**Table 14**). The samples and a protein standard (**Table 13**) are loaded using a Hamilton syringe, which is inserted to about 1mm from the well bottom before delivery. The electrophoresis runs at 80 volts for 30 minutes then at 120 volts for 90 minutes.

Chemical	Amount
Tris-HCl, pH 6,8	2,5ml
SDS	0,2g
Glycerin	4ml
β-Mercaptoethanol (always added fresh)	2ml
Bromphenolblau	4mg
Deionized water	1,5ml

Table 12. Probe buffer 4x (10ml)

Protein Standards	Amount
Kaleidoscope Polypeptide Standards	10μl
Precision Plus Protein Standards	5 μl

Table 13. Protein standards

Chemical	Amount
Tris-Base	15.5 g
Glycin	72.0 g
SDS(10%)	50 ml
make to 5 litres with deionized water	

Table 14. Electrode buffer (5l)

4.8.4 Protein transfer

The transfer Step (Western blot) involves moving and immobilizing the proteins from the SDS- polyacrylamide gel to a nitrocellulose membrane as to be more accessible and quantitatively detected. The 1st antibody binds specifically to the proteins and the 2ry antibody (labelled with horseradish peroxidase HRP) binds to it. The 2ry antibody can be detected visually through the conversion of a substrate to a colored precipitate at site of antibody binding.

The membrane and the filter paper are cut 8.5cm x 5.3cm and soaked with the fibre pad and gel in transfer buffer (**Table 15**). The gel sandwich in Mini Trans-Blot Cell Assembly

(Bio-Rad) is prepared in the following order: cassette with the white side down, fibre pad, 2 filter papers, membrane, gel, 2 filter papers, and fibre pad.

Chemical	Amount
Electrode buffer	500ml
Methanol	200ml
Deionized water	300ml

Table 15. Transfer buffer (1l)

Gloves are used to prevent contamination and air bubbles are removed before the cassette is firmly closed. The cassette is placed in module and the tank is filled completely with buffer. The tank is placed in ice and a standard stir bar is added as to maintain an even buffer temperature and ion distribution in the tank. The blot is run for 2 hours at 100 volts using a powerpac power supply (Bio-Rad). Afterwards, the membranes are placed in glass bowls, stained with Ponceau S and scanned.
Then they are washed with PBS-Tween (0.05%) (**Table. 16**) till the stain disappears.
The nitrocellulose membranes are blocked for immunolabeling by overnight incubation at 4°C refrigerator or 1 h at room temperature using 20 ml 3% or 5% (depending on the antibody) non fat dry milk in PBS-Tween (0.05%).

Chemical	Amount
PBS (phosphate-buffered saline)	9.55g
Tween-20	500µl
make to 1 litre with deionized water	

Table 16. PBS-Tween (0.05%)

After incubation with first antibody and washing 3 x 5min. with 10 ml PBS-Tween (0.05%), the membranes are incubated with the second antibody and then washed again 3 x 5min. with 10 ml PBS-Tween (0.05%) and 1 x 5min with 10ml PBS (**Table 17**). 10 ml of the ECL developing solution is prepared and the membranes are incubated for 1 min, placed between 2 transparency films (without air bubbles) and covered with hyperfilm. The exposure time varies from 1 sec. till 2h depending on the signal.

Chemical	Amount
PBS	9.55 g
make to 1 litre with deionized water	

Table 17. PBS

If the membranes have to be incubated with another antibody, they are washed 30 min. with transfer buffer, 10 min. with PBS/Tween(0.05%), blocked overnight and the new first antibody is added the following morning.

4.8.5 Protein staining

4.8.5.1 Ponceau S red stain

The Ponceau S red stain was regularly performed to confirm that equal amounts of protein were loaded in each lane. The nitrocellulose membrane was taken after protein transfer, placed in a glass bowl, 10ml Ponceau were added and then placed over the shaker for 5 minutes. Afterwards the membrane is cleaned from all gel remnants, washed 5 minutes with PBS/Tween (0.05%) over shaker and then scanned.
The stain could be used 4 or 5 times before getting rid of it.

4.8.5.2 Coomasie blue stain

The coomasie blue protein staining was also done frequently as to prove that equal amounts of protein are loaded in each lane. The SDS-Page is taken carefully after complete separation of the denatured proteins by molecular weight using electrophoresis and placed in a glass bowl. 50 ml of coomasie blue solution (**Table 18**) was poured to the gel and left on the shaker overnight. The gel is washed with water-methanol (4:1) solution till the background is clear. The gel is scanned afterwards and the strength of the protein bands is interpreted using Tina (Fuji) computer program.

Chemical	Amount
concentrated coomasie blue	20 ml
Methanol	20 ml
Deionized water	60ml

Table 18. Coomasie blue solution.

4.9 Real-time RT-PCR:

Intracerebral expression of neuroprotective genes of interest was assessed by semi-quantitative two-step real-time RT-PCR using commercially available and custom-made murine-specific primers (Table). Homogenization of brain tissue samples was performed on ice in 2 ml of Qiazol® buffer (Qiagen, Hilden, Germany) per hemisphere, using an “Ultra TURRAX T25 basic“ homogenizer (IKA Werke, Staufen, Germany). RNA was isolated by phenol-extraction and purified using RNeasy® Mini-kits (Qiagen) and RNA concentrations were measured using a spectrophotometer (Bio-Rad, Munich, Germany). The quality of RNA extraction was verified by bioanalyzer (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). From each brain hemisphere, 2 υg RNA were used for generation of cDNA (complementary DNA) templates using random nonamer and oligo-dT16mer primers

(Operon Biotechnologies, Cologne, Germany) with Omniscript® kits (Qiagen), according to the manufacturer's instructions. Real-time RT-PCR was performed using validated commercially available and custom designed primer-probe® (Qiagen) sets (Table 2) and optimized protocols on the Opticon® real-time PCR Detection System (Bio-Rad). To avoid amplification of contaminated genomic DNA, half of the murine primers were designed to hybridize to the 3' end of one exon and the other half to the 5' end of the adjacent exon. All probes were verified by 3% agarose gel electrophoresis. For quantification of gene expression levels, GAPDH amplicons were generated and used as house-keeping internal control gene. Relative gene expression levels are expressed in relation to the corresponding GAPDH gene expression levels.

4.10 Immunohistochemistry

For assessment of neuronal morphology, integrity, and apoptosis, extracted mouse brains were snap-frozen in liquid nitrogen, embedded in OCT compound (Sakura Finetek, Torrance, CA) and stored at -80^{o}C until used for analysis. Six to eight-micrometer thick coronal tissue sections were cut with a cryostat at -20°C. For immunohistochemistry, slides were fixed in acetone and then analyzed by a standard biotin/avidin/peroxidase technique with DAB (diaminobenzidine)-tetrahydrochloride as chromogen (Vector, Burlingame, CA), as previously described [191, 309]. The following primary antibodies were used as cell-markers: monoclonal anti-NeuN, at a titrated dilution of 1:2,000 (Chemicon, Hampshire, UK) for neurons; polyclonal rabbit anti-GFAP (glial fibrillary acidic protein), 1:100 (Shandon Immunon, Pittsburgh, PA, USA) for astrocytes; monoclonal rat anti-CD11b, 1:100, (Accurate Chemical, Westbury, NY, USA) for microglia; Non-immunized IgG (Vector) was used as negative control at equal dilutions as the omitted specific antibody.

4.11 TUNEL

To determine the extent of intracerebral neuronal cell death, TUNEL histochemistry was performed using a "Fluorescein *In Situ* Cell Death Detection Kit" (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions, as previously described [96]. Briefly, slides were dried for 30 min followed by fixation in 10% formalin solution at RT. After washing in PBS (three times for 3 min), sections were incubated in ice-cold ethanol-acetic acid solution (2:1) for 5 min at -20°C. Thereafter, they were washed in PBS and incubated in a permeabilization solution with 3% Triton X-100 in PBS for 60 min at RT, then incubated with the TdT enzyme in a reaction buffer containing fluorescein-

dUTP for 90 min at 37°C. Negative control was performed using only the reaction buffer without TdT enzyme. Positive controls were performed by digesting equal brain sections with DNase grade I solution (500U/ml; Roche) for 20min at RT and always kept separate from the other samples thereafter. After labelling, the sections were washed again in PBS and to visualize the unstained (TUNEL-negative) cells, the sections were covered with Vectashield® mounting medium for fluorescence with DAPI (Vector). All samples were evaluated immediately after staining using an Axioskop 40 fluorescence microscope (Zeiss, Germany) at 460 nm for DAPI and 520 nm for TUNEL fluorescence and analyzed by Alpha digi doc 1201 software (Alpha Innotech, San Leandro, CA, USA).

5. Results

5.1 Neurological outcome

With regard to the neurological outcome, *Crry-Ig*-treated mice showed a significantly attenuated neurological impairment for up to 7 days after trauma compared to vehicle-injected control mice, as determined by a standardized 10-parameter NSS ($P < 0.05$ *vs.* vehicle-injected TBI mice, repeated measures ANOVA (analysis of variance), (**Fig. 11**).

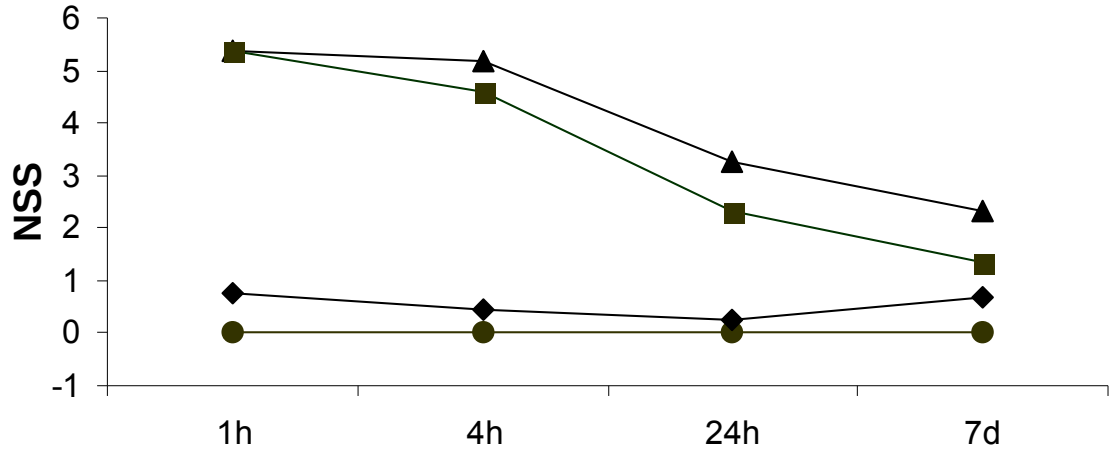

Figure 11. *Crry-Ig* mediates improved neurological recovery after experimental TBI in mice[191]**.** Based on a standardized 10-parameter *Neurological Severity Score* (NSS), *Crry-Ig*-treated mice showed a significantly attenuated neurological impairment from 4h to 7 days after trauma compared to vehicle-injected control mice. The scores were evaluated by two independent investigators who were blinded about the groups. Data are shown as median levels of n=6 mice per group and time-point, except for "nil" controls, n=3 per time-point. *$P < 0.05$, *Crry-Ig vs.* vehicle; repeated measures ANOVA.

In addition, when the individual tasks of the NSS (as depicted in **table 5**) were split up for analysis, the percentage of mice failing single task performances was significantly higher in the vehicle- as opposed to the *Crry-Ig*-treated groups at all time-points assessed[191]. A representative example is shown for the task "beam walk 3 cm" in **Fig. 12**.

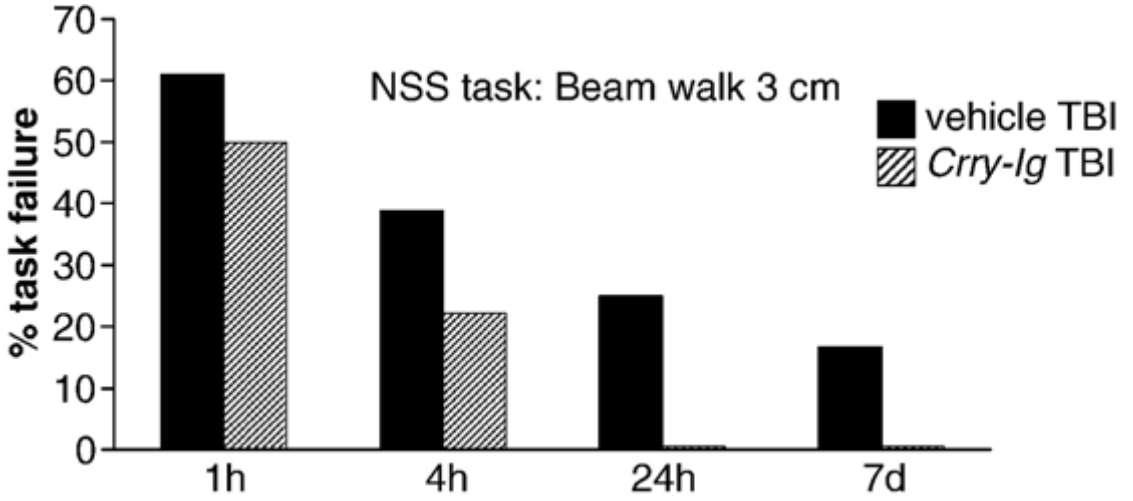

Figure 12. Single Task Performance [191]. This figure shows representative results of differences in single task performance between the vehicle and *Crry-Ig*-injected groups, as shown for the individual task "beam walk 3 cm". Graph bars represent the percentage of mice which failed the task, as determined by two independent, blinded investigators. TBI; traumatic brain injury.

5.2 Complement regulatory genes

Intracerebral expression of neuroprotective genes of interest was assessed by quantitative real-time RT-PCR analysis of brain tissue homogenates using mouse-specific primers (**Table 19**) [191]. Baseline expression of the complement regulatory genes for C1-Inh, CD55, and CD59 was determined in brain homogenates from untreated normal male mice of the C57BL/6 strain ("nil" group, *n*=3 per gene, **Fig. 13**).

	Gene ID at NCBI *	GenBank Accession No.	Length of amplicon	Primer sequence	Probe Sequence	Order No. Qiagen #
GAPDH¶	14433	NM_008084	136 bp	commercially available Genexpression Assay QuantiTect Mm_GAPD		241012
Bcl-2	12043	NM_009741 NM_177410	118 bp	commercially available Genexpression Assay QuantiTect Mm_Bcl-2		241118
CD55	13136	NM_010016	96 bp	CAGTTAAAGTTTCAGC AACCCACTGTGTTAGG CTCTCCTTTGT	CCAAGACAACAGT*ACG	custom made
CD59a	12509	U60473 NM_007652	124 bp	GACTCTAAGATTGCAG ATTTGGGAAACAGTGG TAGCATGTGAGG	TGGCTGTGT*T*CTGT*T*C	custom made
C1-Inh	12258	NM_009776	134 bp	AACTTAGAACTCATCAA CACCTGTTATCTTCCAC TTGGCACTC	ACACCTGCCTCGTCCT	custom made

Table 19. Murine primer sequences used for real-time RT-PCR analysis of intracerebral gene expression [191].

* NCBI = National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/genome/guide/mouse/)
# www.qiagen.com
¶ housekeeping gene

In sham-operated control mice (*n*=6 per gene and time-point), a slight but non-significant increase of C1-Inh expression was noted between 4 hours to 7 days (*P* >

0.05 *vs.* "nil", unpaired Student's *t*-Test; **Fig. 13**). In contrast, the sham procedure led to a significant upregulation of the complement regulatory genes for CD55 and CD59, as compared to baseline expression ($P < 0.05$ *vs.* "nil"; **Fig. 13**)[191]. Experimental brain injury and vehicle control injection at 1h (400 µl PBS) induced a further up-regulation of CD59 mRNA expression within 4 h after trauma ($P < 0.05$ *vs.* sham; **Fig. 13**), but not at later time-points. The gene expression levels of CD55 were not altered at any time-point in the trauma/vehicle group, compared to the sham cohort ($P > 0.05$) [191]. In contrast, the inhibitor of the classical pathway of complement activation, C1-Inh, showed significantly increased mRNA levels at all time-points up to 7 days in the trauma/vehicle group, compared to sham-operated mice ($P < 0.05$; **Fig. 13**)[191].

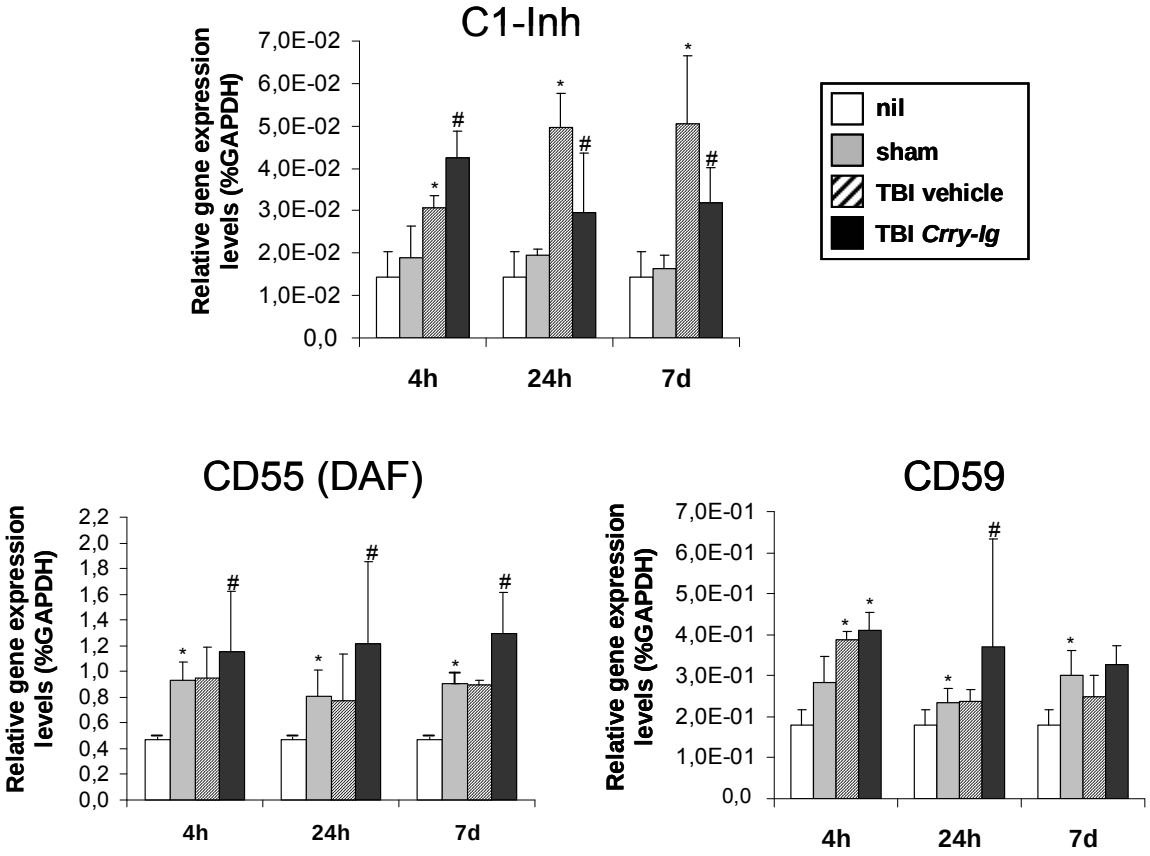

Figure 13. Regulation of posttraumatic intracerebral gene expression by *Crry-Ig*, as determined by quantitative two-step real-time RT-PCR [191]. RNA was extracted from homogenized murine brains at defined time-points. The murine primers for the complement regulatory genes C1-Inh, CD55, and CD59 are shown in table 2. The technique for real-time RT-PCR analysis is described in detail in the *Methods* section. Data are shown as mean levels ±SD from $n=3$ per gene in the "nil" group and $n=6$ per gene and time-point in all other groups. $^{*}P < 0.05$, sham *vs.* "nil" and trauma/vehicle *vs.* sham; $^{\#}P < 0.01$, trauma/*Crry-Ig vs.* trauma/vehicle; unpaired Student's *t*-Test. TBI, traumatic brain injury.

Mice injected with 1 mg *Crry-Ig* one hour after trauma showed a significant up-regulation of C1-Inh and CD55 gene expression levels by 4h after head injury, as compared to vehicle-injected controls ($P < 0.01$), whereas CD59 mRNA levels were not significantly elevated at 4h ($P > 0.05$)[191]. After 24h, both CD55 and CD59 genes showed a significant increase in the *Crry-Ig* group ($P < 0.01$ *vs.* vehicle control; **Fig.13**). The upregulation of CD55 and CD59 genes persisted from 24 h to 7 days. In contrast, C1-Inh mRNA levels were significantly suppressed at 24h in *Crry-Ig*-injected TBI mice, compared to the vehicle-injected animals ($P < 0.01$; **Fig.13**) [191]. A repeated *Crry-Ig* injection at 24h led to a continued down-regulation of C1-Inh mRNA for up to 7 days after trauma ($P < 0.01$ *vs.* vehicle control; **Fig.13**).

5.3 Regulation of Bax and Bcl-2 expression in injured brains

With regard to intracerebral expression and regulation of the mitochondrial anti-apoptotic Bcl-2 gene, a slight but non-significant increase in mRNA levels was noted in sham-operated animals between 24 hours to 7 days, as compared to constitutive levels in normal mice ($P > 0.05$, unpaired Student's *t*-Test; **Fig.14A**)[191]. Bcl-2 gene expression had a trend of increase at 4h and 24h and was significantly increased at 7 days in the trauma/vehicle group (after a repeated injection at 24h), compared to sham-operated animals ($P < 0.05$; **Fig.14A**). After injection of 1 mg *Crry-Ig* one hour after trauma, a significant up-regulation of Bcl-2 mRNA was detected by 4h after trauma, as compared to vehicle-injected controls ($P < 0.01$).[191] At later time-points, Bcl-2 gene expression levels remained elevated in the *Crry-Ig vs.* vehicle control group, albeit at non-significant levels (*t*=24h and 7 days, $P > 0.05$; **Fig.14**)[191].

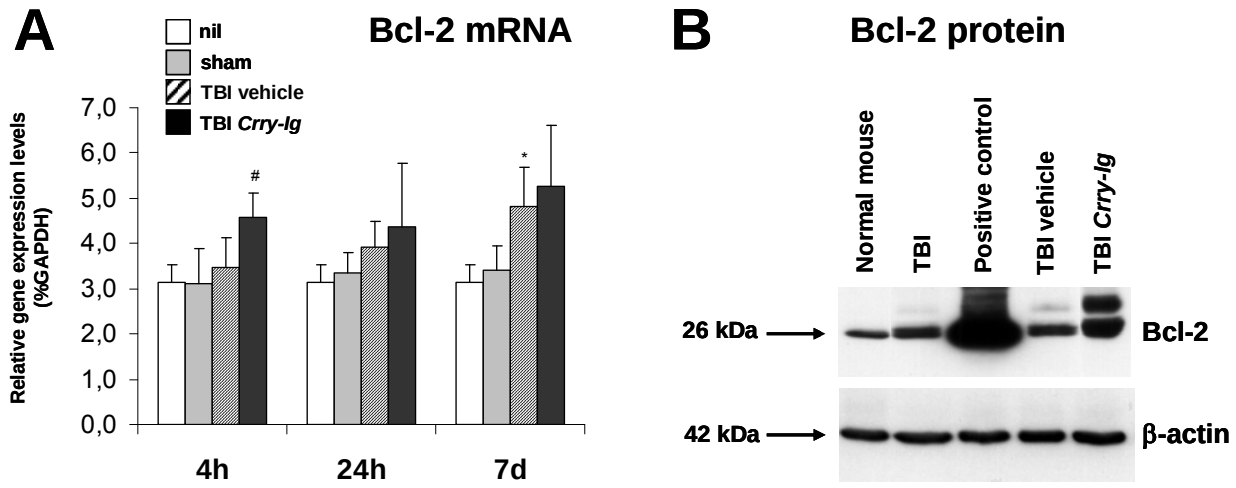

Figure 14. Regulation of the mitochondrial anti-apoptotic mediator Bcl-2 at the mRNA and protein level, as determined by real-time RT-PCR (A) and Western blot analysis (B) [191]. RNA was extracted from homogenized murine brains at defined time-points. The primers used for the murine Bcl-2 gene are shown in table 2. Data are presented as medians ±SD from *n*=3 per gene in the "nil" group and *n*=6 per gene and time-point in all other groups. **P* < 0.05, TBI vehicle *vs.* sham; #*P* < 0.01, TBI *Crry-Ig vs.* TBI vehicle; unpaired Student's *t*-Test. Western blot analysis was performed on murine brain homogenates using a specific monoclonal anti-mouse Bcl-2 antibody and a non-radioactive chemoluminescence assay, as described in the *Methods* section. Equal protein amounts (60 μg per lane) were loaded on SDS-PAGE and consistent blotting was confirmed by Ponceau staining (not shown) or by control blotting with β-actin. Western blot experiments were performed in duplicate in order to ensure reproducibility of the results. TBI, traumatic brain injury. Positive control: Bcl-2 transfected melanoma cells.

By Western blot analysis, Bcl-2 protein levels were detected at constitutive levels in normal untreated mice (**Fig.14B**). Experimental TBI alone or in combination with vehicle injection (400 μl PBS i.p.) induced slightly increased intracerebral Bcl-2 protein levels, compared to normal mouse brain (**Fig.14B**) [191]. In contrast, the injection of *Crry-Ig* led to a strong induction of Bcl-2 protein in the injured mouse brain, as apparent in **Fig.14B**. In addition to the specific Bcl-2 band, which is confirmed by the positive control using Bcl-2 transfected melanoma cells (**Fig.14B**), a presumptive phosphorylation product splice of Bcl-2 [329] is detectable above the 26 kDa band in the TBI brains, with clearly increased levels after *Crry-Ig* injection, compared to TBI and vehicle injection or to TBI alone (**Fig.14B**) [191]. The intracerebral Bcl-2 protein levels were higher in the left hemisphere, which received the direct trauma, (**Fig.15**) than the right hemisphere. The intracerebral Bcl-2 protein levels were higher in the left hemisphere, which received the direct trauma, (**Fig.15**) than the right hemisphere.

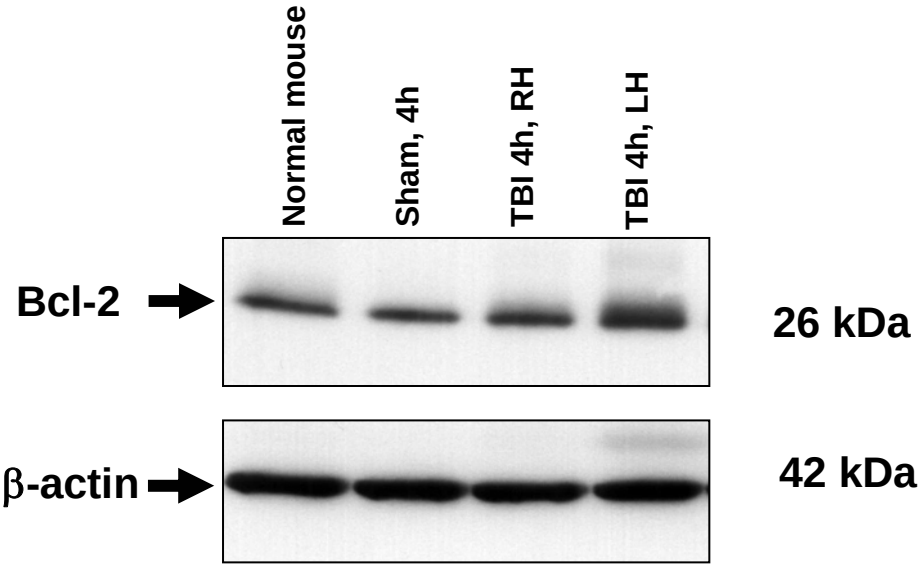

Figure 15. Difference in Bcl-2 expression between left (LH) and right (RH) hemisphere 4h after TBI.

After injection of Crry-Ig after trauma, the level of Bcl-2 protein is relatively increased in comparison to vehicle injected mice by 4h and 7 days, but significantly increased by 24h (**Fig.16**). Nevertheless, sham operated mice had a lower level of Bcl-2 protein at all time points.

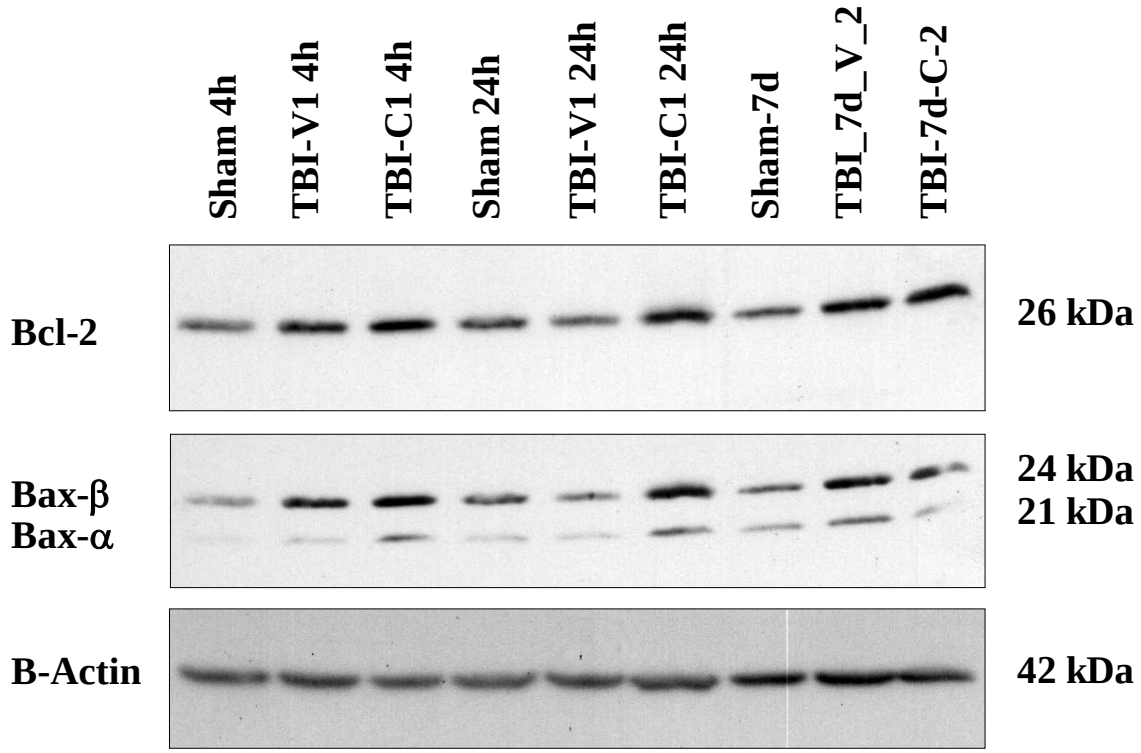

Figure 16. Bcl-2 and Bax expression at different time points (t=1h, 24h and 7 days), Crry-Ig vs. vehicle and sham.

The Bcl-2 level was lower in normal mouse brain (not injected nor operated) than in Sham operated mice (**Fig.17**). It tended to increase by 4h after trauma and was up-regulated after Crry-Ig injection by 4h and 24h. In serum, the level of Bcl-2 was higher in

normal mouse than in sham-operated animals, where the level was high after trauma by 4h in traumatized mice, vehicle as well as Crry-injected mice (**Fig.17**). By 24h the level of Bcl-2 sank in vehicle and Crry-injected mice.

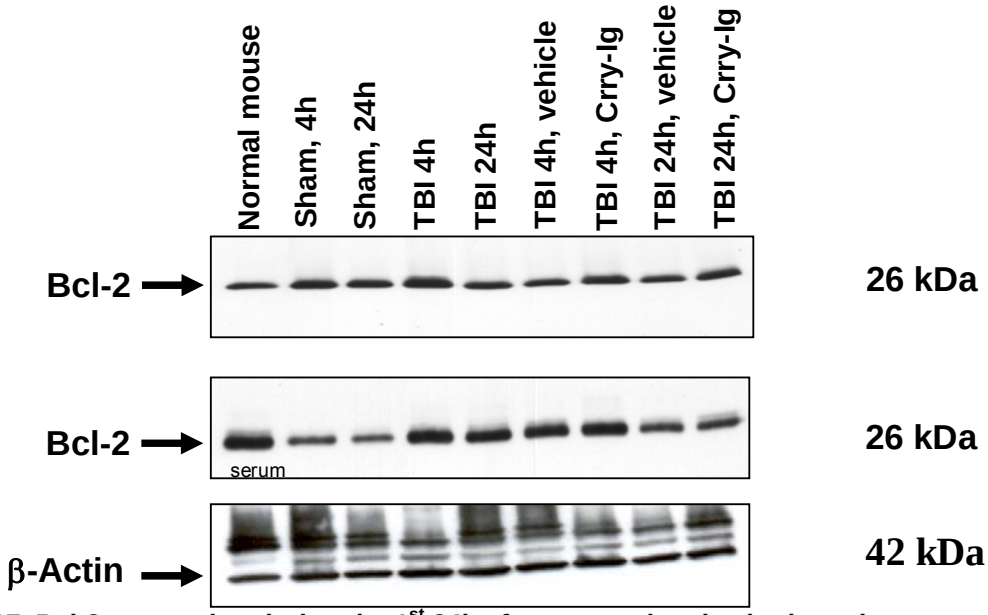

Figure 17. Bcl-2 expression during the 1st 24h after trauma in mice brain and serum

In n=2, we could see that the level of Bcl-2 in serum was again higher in Trauma by 4h than by 24h in Crry-Ig injected mice (**Fig.18**). The level of Bcl-2 raised by 7 days post-trauma, where the highest level was in Sham operated mice and the lowest level was in Crry-Ig injected mice.

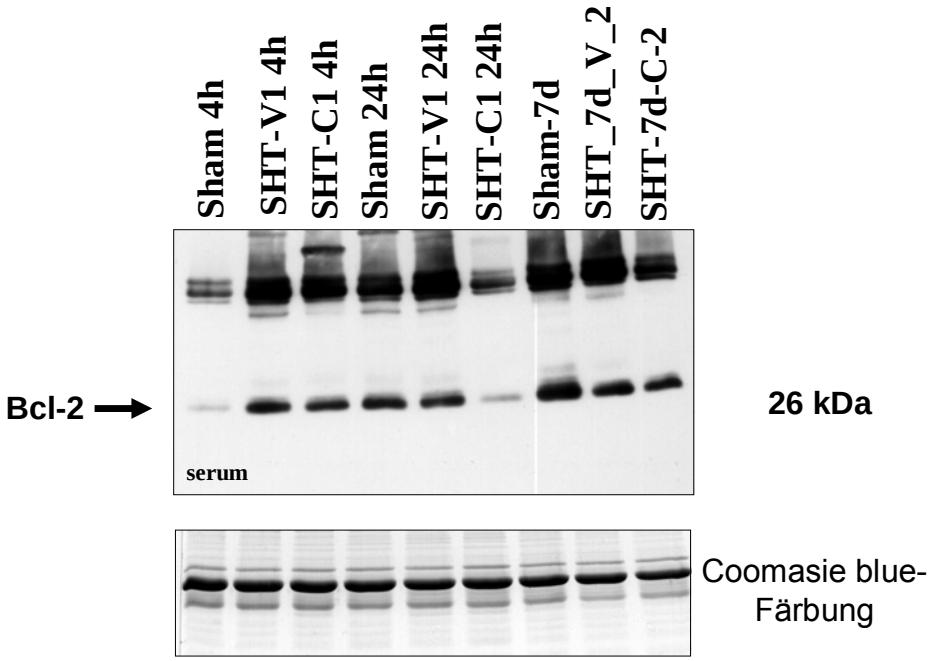

Figure 18. Level of Bcl-2 in serum (t=4h, 24h and 7days)

Bcl-2 level showed a trend to increase after injection of Crry-Ig by 4h (**Fig.19A**) vs. Vehicle injected mice, where the biggest difference was by 24h. 4 weeks post-trauma, the level of Bcl-2 is slightly higher in Vehicle-injected mice vs. Crry-Ig injected mice (**Fig.19A**).The strength of the signal produced by Bcl-2 and β-Actin was analyzed using Tina 2.09 software (Raytest, Straubenhardt, Germany) and the relative expression of Bcl-2 in relation to β-Actin was calculated and displayed (**Fig.19B**).

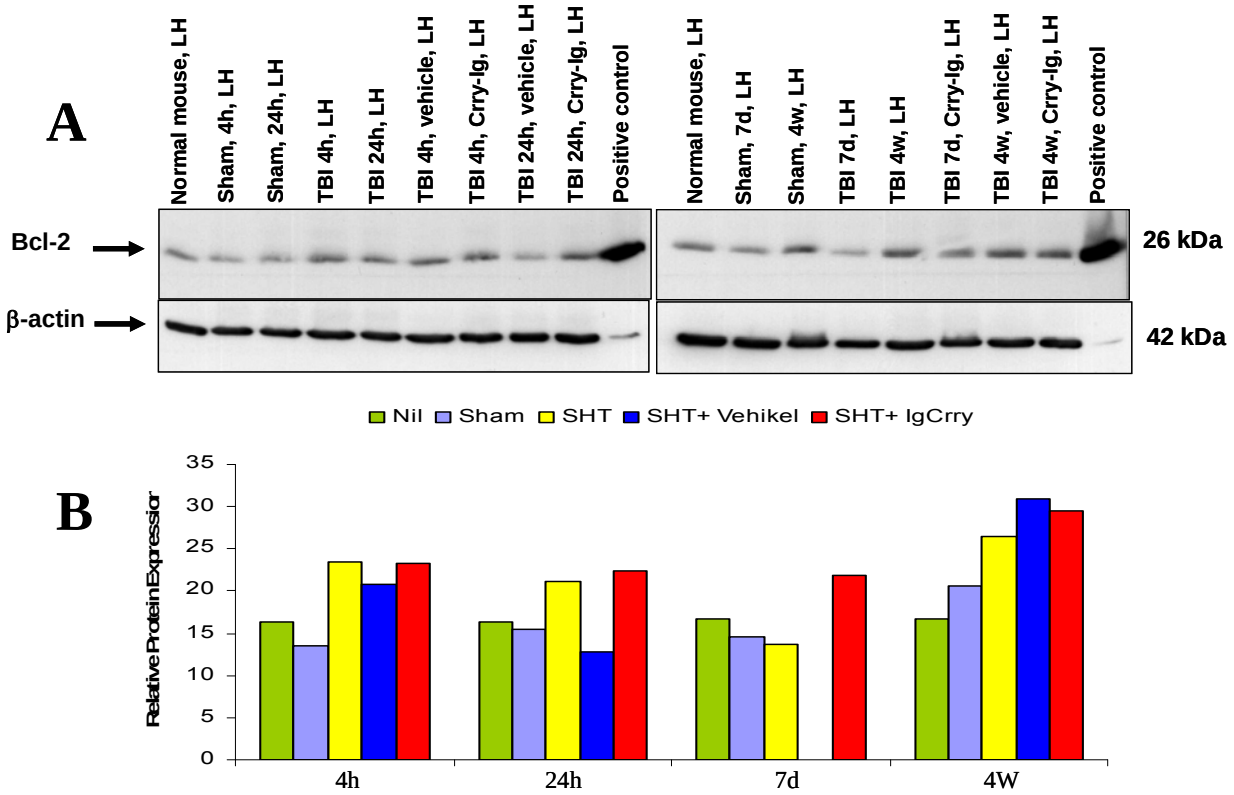

Figure 19. Expression of Bcl-2 protein level (t=4h, 24h, 7days and 4 weeks) and the relative expression of Bcl-2 in relation to β-Actin.

The level of Bax-α protein in brain was determined using Western Blot. The lowest level was in sham-operated mice at all time points. By t=4h the level of Bax increased slightly after Vehicle injection but showed a higher up-regulation after injection of Crry-Ig (**Fig. 16**). By t=24h, the level of Bax-α was notably elevated after Crry-Ig injection in comparison to vehicle injection, which was as well detected 7days post-trauma (**Fig. 16**). The Bax-α level was lower in normal mouse brain (neither injected nor operated) than in Sham operated mice (**Fig.20**). It tended to increase by 4h after trauma and was up-regulated after Crry-Ig injection by 4h and 24h. In serum, the level of Bax-α couldn't be detected (**Fig.20**).

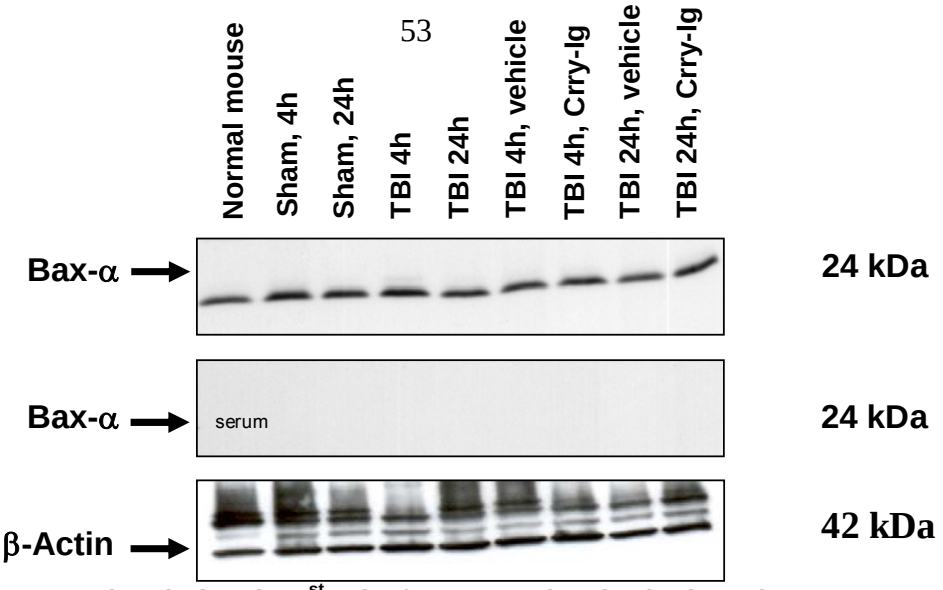

Figure 20. Bax-α expression during the 1st 24h after trauma in mice brain and serum

Bax-α level showed a trend to increase after injection of Crry-Ig by 4h (**Fig. 21A**) vs. Vehicle injected mice, where the biggest difference was by 24h. 4 weeks post-trauma, the level of Bax-α is slightly higher in Vehicle-injected mice vs. Crry-Ig injected mice (**Fig. 21A**).The strength of the signal produced by Bax-α and β-Actin was analysed using Tina 2.09 software (Raytest, Straubenhardt, Germany) and the relative expression of Bax-α in relation to β-Actin was calculated and displayed (**Fig. 21B**).

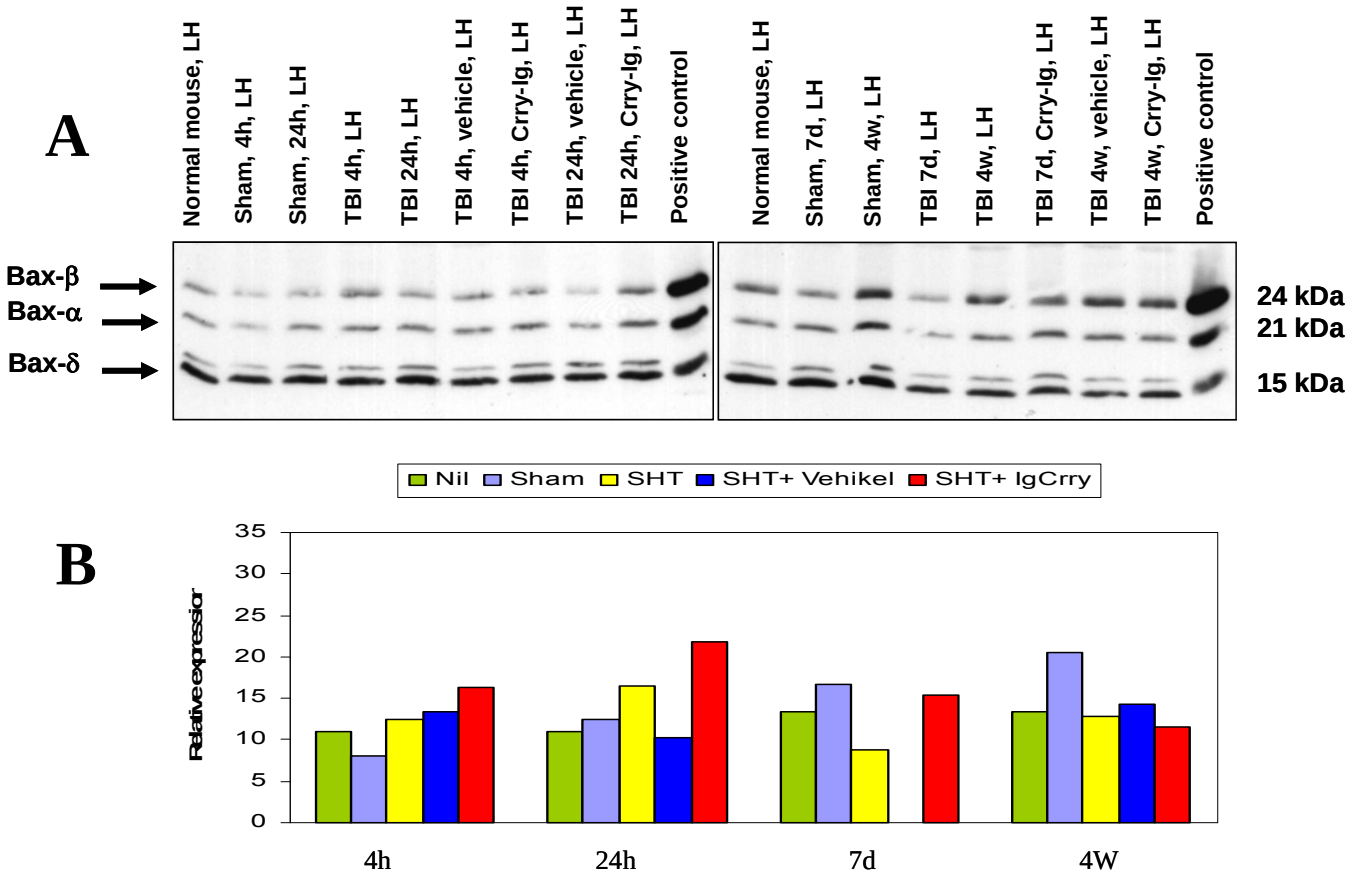

Figure 21. Expression of Bax-α protein level (t=4h, 24h, 7days and 4 weeks) and the relative expression of Bax-α in relation to β-Actin.

A slight but non-significant increase in mRNA levels was noted in sham-operated mice between 24 hours to 7 days in comparison to constitutive levels in normal mice (**Fig.22**). The Bax gene expression tended to remain constant at different time points (t= 4h, 24h and 7days) in the vehicle group (**Fig.22**). The Bax mRNA was up regulation after injection of Crry at the different time points in comparison to Vehicle injected mice

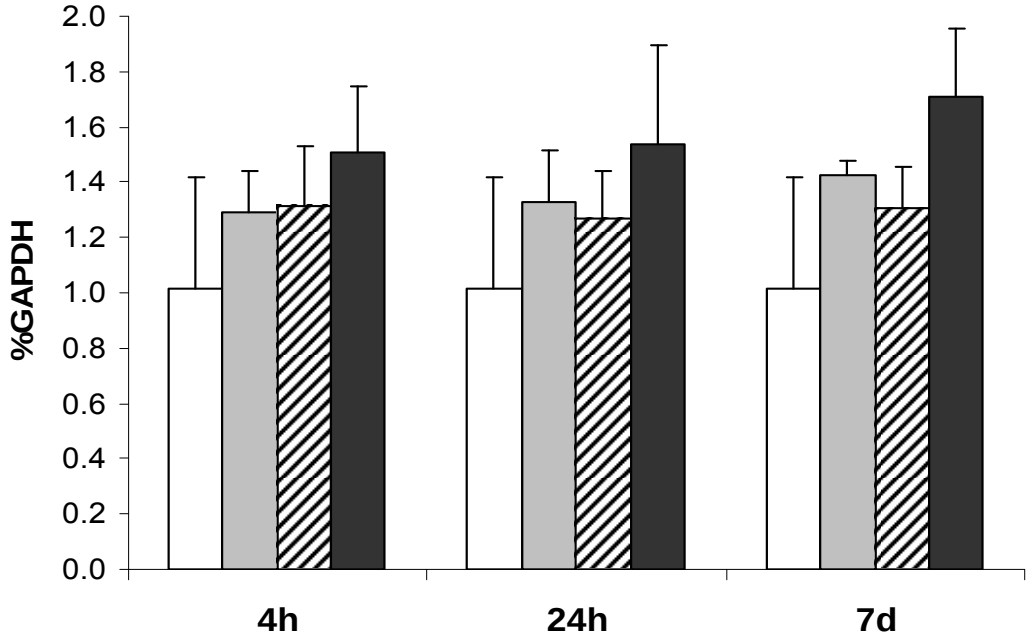

Figure 22. Regulation of the mitochondrial pro-apoptotic mediator Bax at the mRNA level, as determined by real-time RT-PCR

5.4 Posttraumatic neuronal cell death

Morphologically, hippocampal neurons in the Ammon's horn (*Cornu ammonis*; CA) layers 3 and 4 of the ipsilateral hemisphere showed a massive destruction of the microarchitecture and loss of normal morphology, such as the polygonal large body shapes of pyramidal neurons, within 4 hours after trauma in the vehicle-injected mice, as compared to "sham"-operated controls (**Fig. 23, middle panels**) [191]. In contrast, the *Crry-Ig*-injected mice showed clear histological signs of neuronal protection, since the highly vulnerable pyramidal neurons in the CA3/CA4 sublayers of the hippocampus were restored to similar morphological features as in the "sham"-operated group (**Fig. 23**) [191].

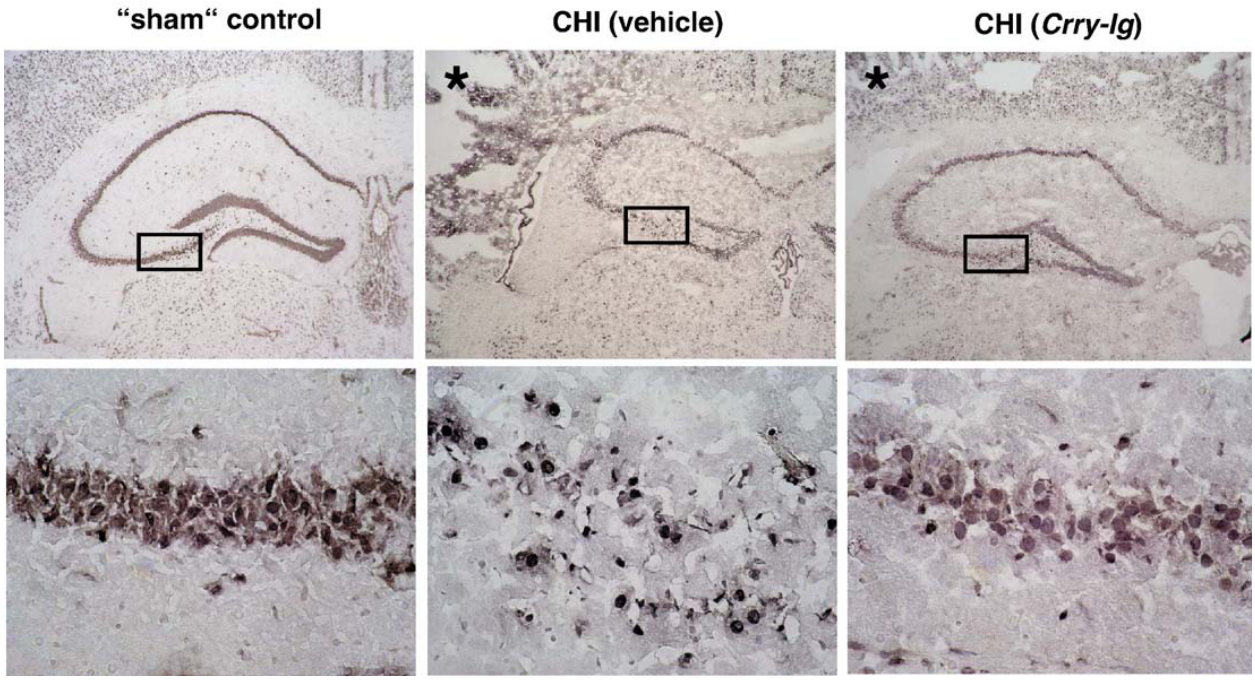

Figure 23. Protective action of Crry-Ig against posttraumatic neuronal cell death in hippocampal CA3/CA4 regions [191]. Male C57BL/6 mice (n = 3 per group) were killed 4 h after "sham" operation (left panel) or after i.p. injection 1 h after CHI with 400 µl PBS alone (vehicle control; middle panel) or with 1 mg Crry-Ig in 400 µl PBS (right panel). Determination of neuronal morphology and microarchitecture was performed by immunohistochemical staining of coronal cryosections with a monoclonal anti-NeuN antibody, as described in the Methods section. While "sham"-operated animals showed a normal neuronal morphology in the hippocampus (left panel), CHI resulted in a clear reduction and architectural destruction of the pyramidal neuron population mainly in the Ammon's horn CA3/CA4 regions, as shown for a representative vehicle-injected CHI mouse (middle panel). In contrast, the treatment with Crry-Ig after trauma clearly attenuated the neuronal cell loss in the corresponding hippocampal sublayers and partially restored the neuronal cell layer morphology in the CA3/CA4 sublayers (right panel). The bottom figures are a 10× magnification of the drawn insert in the upper figures (original magnifications: 40× vs. 400× in upper vs. bottom microphotographs, respectively). The asterisk marks the contusion zone in the left cortical hemisphere of the brain-injured animals.

The extent of neuronal cell death is shown to be limited after the injection of Crry-Ig, where the number of TUNEL positive cells in the hippocampal CA3 region was much less by t=4h and 24h than in Vehicle injected mice (**Fig.24**).

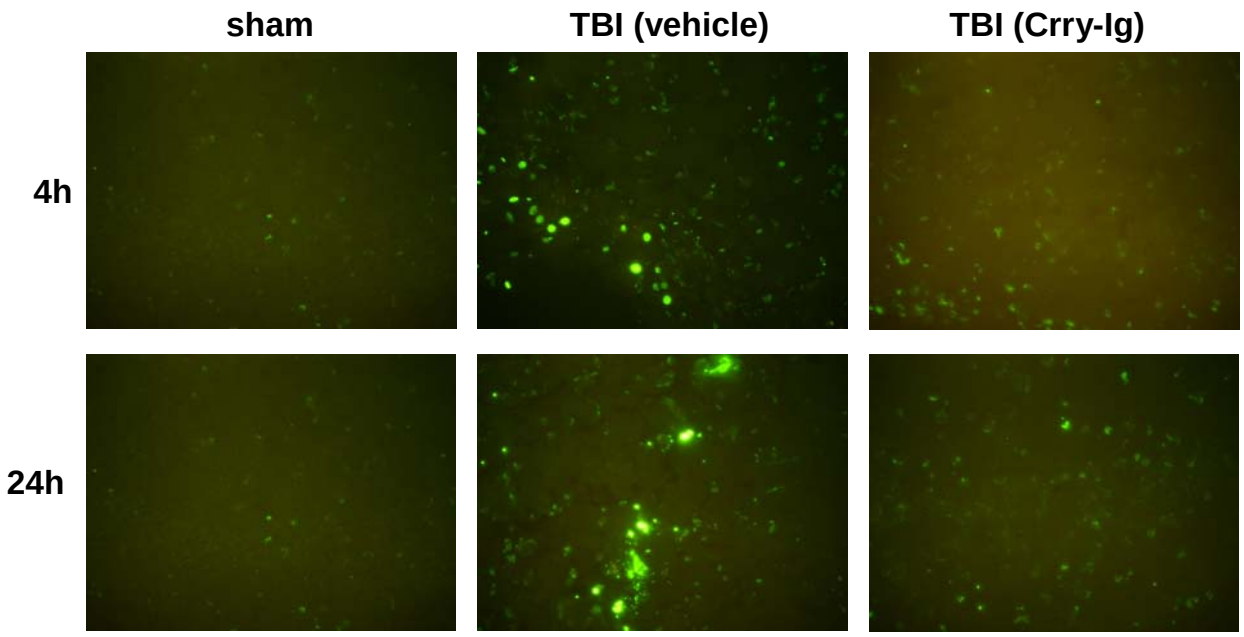

Figure 24. Posttraumatic neuronal cell death in hippocampal CA3 region in sham operated mice and mice with Traumatic brain injury after vehicle and Crry-Ig injection, as determined by TUNEL histochemistry. The magnification is 400x

In the ipsilateral cortical region of injured hemisphere, the Crry-Ig injection prevented the extension of neuronal cell death in comparison to the vehicle injected mice by t=4h and 24h (**Fig.25**), where the number of TUNEL positive cells was less.

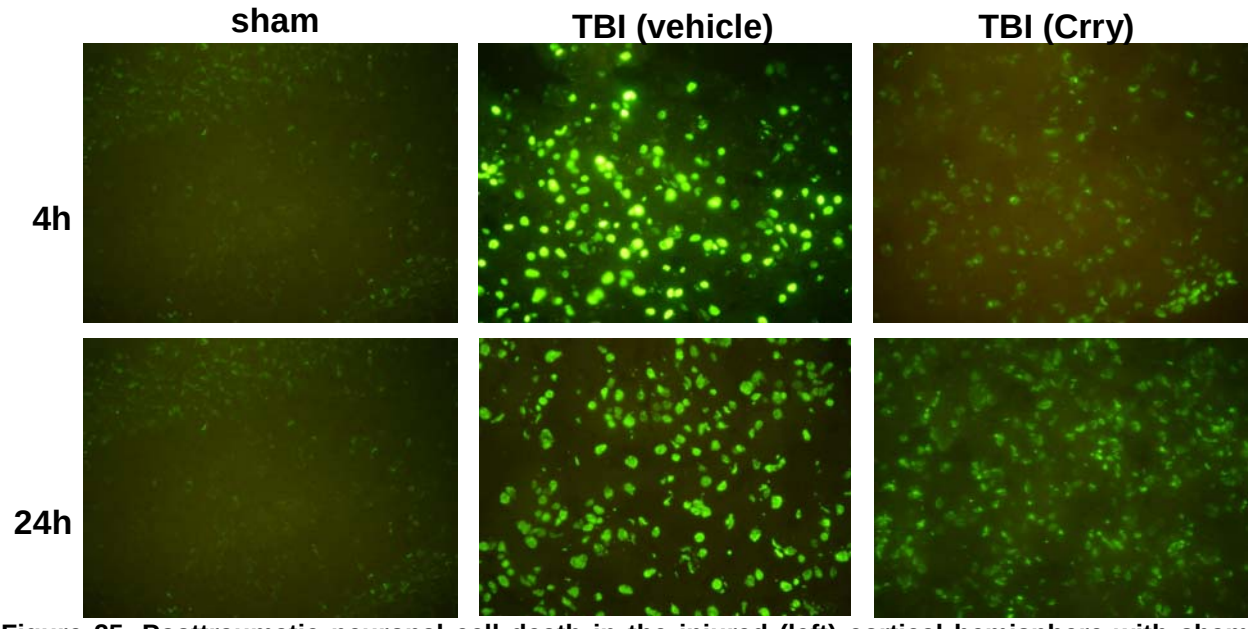

Figure 25. Posttraumatic neuronal cell death in the injured (left) cortical hemisphere with sham operated mice and mice with Traumatic brain injury after vehicle and Crry-Ig injection, as determined by TUNEL histochemistry. The magnification is 400x.

6. Discussion

Neuroprotection from complement-mediated inflammatory damage to the brain represents a prevailing topic of research in neuroinflammatory and neurodegenerative diseases [56, 310-315]. In the present study, we assessed the effect of pharmacological complement inhibition at the C3 convertase level by exogenous administration of murine recombinant *Crry-Ig* on intracerebral gene expression profiles, the neurological outcome[191], regulation of apoptotic proteins and state of hippocampal neurons after experimental TBI. We demonstrate here for the first time, to our knowledge, that the posttraumatic pharmacological blocking of complement activation by *Crry-Ig* leads to a significantly increased neurological recovery after CHI in mice, as compared to vehicle-injected control animals [191]. Furthermore, the neuronal subsets with highest vulnerability to head injury in the hippocampal layers [299, 300, 316, 317] were protected by *Crry-Ig* administration, as opposed to vehicle-injected brain-injured mice. The analysis of neuroprotective gene expression patterns in injured brains revealed that the systemic injection of *Crry-Ig* mediates an upregulation of complement regulatory genes (C1-Inh, CD55, CD59) and of the potent mitochondrial anti-apoptotic mediator Bcl-2, both at a gene and protein level, with differential expression kinetics of these mediators during the first 7 days after trauma[191]. The pro-apoptotic mediator Bax was up-regulated but to a lesser extent than Bcl-2 with a neuroprotective end effect as detected by anti-NeuN antibody staining and TUNEL histochemistry.

In order to establish a pharmacological model with potential clinical relevance, we chose a peripheral route of administration (i.p.) during the “time window of opportunity” at one hour after trauma. This timing correlates with the potential for early therapeutic intervention in TBI patients [5] and with the time-frame of early BBB dysfunction in the present murine model system, thus allowing for a pharmacological compound to reach the intrathecal compartment after systemic injection, as previously determined for other pharmacological agents [117, 300, 301]. The penetration of the *Crry-Ig* compound into the intrathecal compartment after i.p. injection in mice was recently confirmed in a different setting of experimental neuroinflammation based on a model of murine lupus cerebritis [296]. The strategy of a repeated injection at 24h was previously established and shown to be effective for other neuroprotective drugs in this murine model[300, 301]. In the present study, the injection of 1 mg *Crry-Ig* in 400 µl PBS at 1h and 24h after trauma led to a significant complement inhibition in serum within 4h to 7 days compared to vehicle-injected controls. Thus, this dose regimen – which is in the titrated

range from previous studies on different models of inflammation in the mouse[296-298] – is functional in the *in vivo* setting of trauma-induced neuroinflammation in our model of experimental TBI.

The extent of posttraumatic neurological impairment was determined in a blinded fashion using a previously characterized 10-parameter score (NSS) [96, 117, 300, 304]. This score has been shown to correlate with the severity of brain injury and the volume of injured brain tissue, as determined by MRI and histological analyses [304]. In the present study, the administration of *Crry-Ig* at 1h and 24h after head injury resulted in a significantly improved neurological outcome of brain-injured mice for up to 7 days, as compared to vehicle-injected control animals. This finding is consistent with previous data from the same model system, where transgenic mice with astrocyte-targeted overexpression of the *Crry* gene had a significantly decreased NSS within 1h to 4 weeks after trauma, as compared to brain-injured wild-type littermates[192]. Thus, our present results provide a pharmacological extension of the “baseline“ data derived from the transgenic model [192] and affirm the feasibility of complement inhibition by *Crry-Ig* in experimental neurotrauma.

In order to assess the potential molecular mechanisms underlying the *Crry-Ig*-mediated beneficial effects on neurological scores, we further investigated the expression and regulation of candidate protective genes in the injured mouse brain [191]. These include Bcl-2, a crucial anti-apoptotic mediator in the mitochondria [248], as well as selected complement regulatory molecules, such as C1-Inh, a major control molecule of the classical pathway of complement activation, DAF/CD55, a membrane-bound inhibitor at the C3 level of complement activation, and CD59, a “key“ regulator of terminal complement pathway MAC/C5b-9 deposition in brain tissue [318-320] [319-324]. Furthermore, we investigated Bax, a critical pro-apoptotic mediator (Deckwerth 1996) on the gene expression level and protein synthesis and finally the end effect of Crry-Ig on apoptosis and neuronal cell death. We found a differential regulation and differing kinetics of intracerebral gene expression after *Crry-Ig* administration. Interestingly, “sham“ operation alone (i.e. isoflurane anesthesia and scalp incision) led to a significant induction of complement regulatory genes for CD55 and CD59, as compared to baseline constitutive gene expression levels in untreated mice[191]. It was previously shown that complement gene expression in the murine brain is in part affected by basic procedures such as “sham“ operations under anesthesia, as described for C5aR mRNA expression on neurons [309]. Since this effect was not observed for every gene

investigated in the present study, we hypothesize that CD55 and CD59 mRNA‘s have a lower threshold for induction by anesthetic drugs, such as isoflurane, as opposed to the genes for C1-Inh or Bcl-2. The mechanisms of anesthesia- or “sham“-operation-induced gene regulation in the CNS are unclear at present and remain to be further investigated.
After trauma, the genes for C1-Inh and CD55 were significantly upregulated within 4h in the *Crry-Ig*-injected group, as compared to vehicle-injected controls. The CD59 gene showed a slightly differing kinetic with a peak at 24 h, whereas the CD55 gene remained significantly elevated above the vehicle controls for up to 7 days after TBI. In contrast, the C1-Inh gene was significantly suppressed at the later time-points (24h and 7 days) compared to vehicle-injected mice. These data suggest that *Crry-Ig* modulates its anti-complement effects not only by direct inhibition of C3 convertases [325, 326], but also by indirect upregulation of other complement-regulatory genes (C1-Inh, CD55, CD59).
Apoptotic neuronal cell death contributes to secondary neurodegeneration after brain injury [95, 327]. Bcl-2 represents one of the crucial mitochondrial regulators of apoptosis and a potent neuroprotective molecule [248, 328]. In the present study, we detected a posttraumatic upregulation of Bcl-2 mRNA within 7 days, which was further induced in the *Crry-Ig*-treated group as early as 4h after trauma. *Crry-Ig*-mediated increase in intracerebral Bcl-2 expression was furthermore confirmed at the protein level by Western blot analysis. An extra band above the specific 26 kDa Bcl-2 band (**Fig. 14B**) may result from phosphorylation of Bcl-2, a phenomenon which was recently described as a new molecular mechanism of regulation of apoptosis [329]. These data suggest that Bcl-2 may exert beneficial mechanisms of action with regard to protection from complement-induced neuronal apoptosis after brain injury [95, 150].
Bax represents a key pro-apoptotic modulator [248]. Interestingly, we detected as well a relative posttraumatic up-regulation of Bax mRNA within 7days which was further induced in the Crry-Ig injected mice. The synthesis of Bax protein was stimulated posttraumatic as detected by western blot but the Bcl2/Bax ratio was in favor of Bcl-2 having a neuroprotective end effect.
The neuroprotective effect of Crry-Ig was confirmed by the clear histological signs of neuroprotection after staining of hippocampus and cortex with anti-NeuN antibody.
The TUNEL histochemistry showed a limited number of TUNEL positive cells in the Crry-treated mice vs. Vehicle-treated mice, which further proved the defensive effect of Crry-Ig against neuronal cell death and apoptosis.

Studies by other groups have previously addressed the potential of pharmacological complement inhibition in different TBI models [36, 186, 200]. However, these studies should be interpreted with caution due to drawbacks in the experimental designs as well as questions regarding the potential clinical relevance of the results [191]. Specifically, previous models used human complement inhibitors in a rat trauma model due to the lack of species-specific reagents at the time [36]. Furthermore, in these previously published studies the complement inhibitor was injected before the injury, a protocol which cannot be extrapolated into a clinically relevant setting [36, 186, 200]. Finally, in contrast to the standardized fluid-percussion injury model [36, 200] and our model of CHI [117, 299-301], the experimental cryoinjury model [186] does not have a clinical relevance since it does not represent an injury pattern which occurs in TBI patients. The data from the present study, using a species-specific and highly potent complement inhibitor at the level of the C3 convertase of all complement activation pathways, provides a strong rationale for pharmacological complement inhibition in the early phase after head injury and provides the basis for future studies on the exact cellular and molecular mechanisms of neuroprotection mediated by *Crry-Ig [191]*.

One drawback of the present study is the lack of a demonstration of direct *Crry-Ig*-mediated effects in the injured brain. It may well be that the blocking of anaphylatoxin C5a formation by *Crry-Ig* after head injury prevents chemotaxis of potentially neurotoxic peripheral immune cells, such as neutrophils, across the BBB. This was previously shown in a rat model of head injury by the use of sCR1, a human recombinant inhibitor of complement C3 convertases [36]. Thus, whether the observed beneficial outcome of complement inhibition is due to direct or indirect mechanisms of action by *Crry-Ig* remains to be determined in future studies [191].

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8. Abbreviations

aa	amino acid
AD	Alzheimer's disease
ADP	adenose-diphosphate
ANOVA	analysis of variance
Apaf-1	apoptosis activating factor 1
APR	acute-phase response
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
Bcl-2	B-cell leukemia/lymphoma-2
BDCF	brain-derived chemotactic factor
BH	Bcl-2 homology
C	Complement
C1-INH	C1 inhibitor
C3aR / C5aR	C3a receptor / C5a receptor (CD88)
C4-bp	C4-binding protein
CCI	Controlled cortical impact
cDNA	complementary DNA
CHI	closed head injury
CGNs	cerebellar granule neurons
CNS	central nervous system
CPP	cerebral perfusion pressure
CR	complement receptor
CRP	C-reactive protein
Crry	Complement receptor-related protein y
CSF	cerebrospinal fluid
Cyt c	cytochrome c
DAF	decay-accelerating factor (CD55)
DAB	diaminobenzidine
DAI	diffuse axonal injury
DNA	deoxy-ribonucleic acid
EAA	excitatory amino acid
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cells
FasL	Fas (CD95)-ligand
FEASA	Federation of European Animal Science Association
FPI	Fluid percussion injury
GFAP	glial fibrillary acidic protein
GPI	glycosyl-phosphatidyl-inositol
HRP	horseradish peroxidase
ICP	intracranial pressure
Ig-Crry	immunoglobulin G1-Crry
IL / IFN	interleukin / interferon

i.p	intra peritoneal
LH	left hemisphere
MaBP	Mannose-binding protein
MBL	mannose-binding lectin
MAC	membrane attack complex (C5b-9)
MCP	membrane cofactor protein (CD46)
MO	monocytes
MS	multiple sclerosis
NGF	nerve growth factor
NK	natural killer
NO	nitric oxide
NOS	nitrogen oxide synthase
NSS	Neurological Severity Score
Page	polyacrylamide gel electrophoresis
PARP	poly ADP-ribosyl DNA polymerase
PBS	phosphate-buffered saline
PCD	programmed cell death (apoptosis)
PCR	polymerase chain reaction
PI-PLC	phosphatidyl-inositol-specific phospholipase C
PKA	protein kinase
PMN	polymorphonuclear leukocytes
PTP	permeability transition pore
RH	right hemisphere
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse-transcription polymerase chain reaction
SAS	subarachnoid space
SCI	spinal cord injury
sCR1	soluble complement receptor type 1
sCrry	soluble Crry
SDS-page	sodium dodecyl sulfate polyacrylamide gel
SPF	specific pathogen-free
TBI	traumatic brain injury
TdT	terminal deoxynucleotidyl transferase
TNF(R)	tumor necrosis factor (receptor)
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VCP	Vaccinia virus complemet control protein

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